THE ROLE OF *SOX4* IN KIDNEY DEVELOPMENT

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ABSTRACT

Over the last decade the number of Canadians with end-stage kidney disease (ESKD) has steadily risen and there are at present more than 35,000 Canadians living with ESKD. Currently only three treatment options are available to replace the blood filtering function performed by the kidneys: hemodialysis, peritoneal dialysis and kidney transplant. A better understanding of the mechanisms involved in kidney development could hold the key to developing targeted therapies to repair and reverse damage caused by kidney diseases.

The DNA-binding transcription factor Wilms' Tumor Suppressor-1 (WT1) plays an essential role in nephron progenitor cell differentiation during kidney development. In mice, homozygous deletion of *Wt1* results in complete absence of both kidneys. Previously, chromatin-immunoprecipitation coupled to microarray identified all three members of the S*ry-related high-mobility group (HMG) Box (Sox)C* subfamily, namely, *Sox4, Sox11* and *Sox12*, as novel Wt1 target genes that may regulate kidney development *in vivo*. Although *SoxC* genes play master roles in determining neuronal and mesenchymal progenitor cell fate in a multitude of developmental processes, their function in the developing kidney has been, until now, undefined.

All three *SoxC* genes are expressed in the nephrogenic lineages during kidney development. Conditional ablation of *Sox4* in nephron progenitors and their cellular descendants (*Sox4*$^{neprhon-}$ mice) results in early-onset proteinaceous glomerular injury within 2 weeks of birth progressing to end-stage renal failure within 5-9 months. An

updated version of the gold standard modality to accurately, rapidly and cost-effectively quantitates nephron number in embryonic and post-natal mouse kidneys was developed. This approach was used to determine that ablation of *Sox4* in nephron progenitors and their cellular descendants results in a significant reduction in nephron endowment. This reduction in nephron endowment in $Sox4^{neprhon-}$ mice results from the formation of fewer pretubular aggregates, likely as a result of decreased cell cycling of nephron progenitor cells. *Sox4* and *Sox11* were also shown to be dispensable for normal podocyte differentiation and function. Conditional deletion of both of these genes individually as well as in combination in podocytes resulted in no apparent glomerular phenotype after 6 months and does not recapitulate the phenotype observed in $Sox4^{neprhon-}$ kidneys. Analysis of $Sox4^{neprhon-}$ kidneys suggests that the genesis of glomerular injury may be activated parietal epithelial cells (aPEC).

Collectively, the results presented in this thesis demonstrate that *Sox4* plays multiple crucial roles in normal kidney development and function *in vivo.*

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LIST OF ABBREVIATIONS

aPEC	activated parietal epithelial cells
BC	Bowman's capsule
BS	Bowman's space
CAKUT	congenital anomalies of the kidney and urinary tract
CV	coefficient of variability
E	embryonic day
ESKD	end-stage kidney disease
GBM	glomerular basement membrane
GFR	glomerular filtration rate
H&E	hematoxylin and eosin
HMG	high-mobility group
MET	mesenchymal-to-epithelial transition
MIQE	minimum information for publication of quantitative real-time PCR experiments
MM	metanephric mesenchyme
MRI	magnetic resonance imaging
N_{glom}	glomerular number
NG2	neural/glial antigen 2
NPC	nephron progenitor cells
OCT	optimum cutting temperature
P	postnatal day
PAS	periodic acid-Schiff
PBS	phosphate-buffered saline

PEC	parietal epithelial cells
PECAM-1	protein platelet and endothelial cell adhesion molecule 1
PNA	peanut agglutinin
PTA	pretubular aggregates
RT-qPCR	reverse transcription quantitative PCR
RV	the renal vesicle
Sox	Sry-related high-mobility group (HMG) Box
S-SB	S-shaped bodies
TP	the tubular pole
UB	ureteric bud
VP	vascular pole
WT	wild-type
WT1	Wilms' Tumor Suppressor-1

1. General Introduction

The kidney is an essential organ. Its primary function is the removal of waste from the bloodstream as well as the regulation of electrolytes and water. Unlike many other organ systems in the body, such as the skin, intestine, lungs, and liver, which undergo constant cellular turnover (Pellettieri and Sánchez Alvarado 2007), a population of adult stem cells has not as of yet been definitively identified in the kidney that is maintained following the completion of kidney development. The continued identification and understanding of fundamental processes which underlie the development of a functioning kidney is crucial to understand the etiology of diseases and to identify potential therapeutic targets for their treatment.

Nephrogenesis is the production and development of nephrons in the kidney. Following its completion, the number of nephrons present, referred to as nephron endowment, is set for a given individual. Nephron endowment varies greatly within human and animal populations, ranging from 7,000 to 20,000 in mice (Cebrian et al. 2004, Murawski et al. 2010, Zhong et al. 2012), and from 200,000 to 2 million in humans (Hughson et al. 2003, Hoppe et al. 2007, Bertram et al. 2011). Once nephrogenesis is complete (at 34-36 weeks of gestation for humans and postnatal day 7-10 for mice), the number of nephrons will not increase over the course of an individual's life, but may decrease as a result of injury or insult. An increasing body of evidence now links low nephron endowment to poor long-term health outcomes and adult-onset hypertension (Keller et al. 2003, Reyes and Mañalich 2005, Walker et al. 2012).

Because nephrogenesis occurs largely during embryonic development, an increased understanding of the fundamental signaling pathways and events that underlie embryonic kidney development are essential. This knowledge will lay a foundation for new therapies and stem cell-based strategies aimed at kidney regeneration to prevent and reverse the effects of kidney disease. This increased understanding also has the potential to identify better therapeutic targets for the treatment of kidney disease.

1.1 End Stage Kidney Disease and Treatments

1.1.1 Canadian Statistics

According to the Canadian Institute for Health Information, more than 35,000 Canadians (excluding Quebec) were living with end-stage kidney disease (ESKD) in 2014, with 58% of those patients receiving some form of dialysis (CIHI 2016). Over 5,200 new Canadian patients were also diagnosed with ESKD in 2014, a number which continues to rise from year to year. Diabetes was the main cause of ESKD in approximately 36% of newly diagnosed patients (CIHI 2016). Diabetic nephropathy is rapidly becoming the major cause of cardiovascular mortality and ESKD worldwide (Khan and Quaggin 2015). Dialysis represents a substantial financial burden to the Canadian health care system, with the annual cost being approximately $60,000 per patient. Dialysis also has a substantial impact on the overall quality of life of the patient, with standard haemodialysis regiments consisting of several multiple hour sessions per week. It is estimated that over a five-year period, a single kidney transplant saves the Canadian health care system more than $250,000, while also significantly improving the quality of life of the patient. Unfortunately, not all patients are eligible for a transplant and some of those that are can spend years on a waiting list. Between 2005 and 2014,

the waiting list in Canada for kidney transplants remained approximately 2.5 times longer than the number of transplant surgeries performed (CIHI 2016).

1.1.2 Treatment for ESKD

Proteinuria, the presence of abnormal amount of protein in urine, fibrosis and endothelial injury all contribute to ESKD. Left untreated ESKD will lead to coma, seizures and death. There are currently only three treatment options to replace the vital life maintaining blood filtering function performed by the kidneys which is lost during ESKD. The three treatments are i) hemodialysis, ii) peritoneal dialysis and iii) kidney transplant. A fourth option that may be pursued is conservative management, where the focus is on using medication and diet to keep the patient comfortable and to treat the resulting issues of ESKD such as anemia, edema and pain. Although none of the four treatment options focus on repairing damage to the kidneys, they aim to improve the quality of life of the patient. With an ever increasing proportion of ESKD resulting from diabetic nephropathy, a substantial body of research is focused on novel agents aimed at targeting molecular pathways that may arrest rather than simply slow the progression of ESKD (Khan and Quaggin 2015).

Dialysis involves the diffusion of molecules in solution across a semipermeable membrane along a concentration gradient. The primary purpose of hemodialysis (dialysis of the blood) is the removal of metabolic waste and excess fluid from the circulating bloodstream in order to restore the intracellular and extracellular fluid environment that is present when kidney function is normal (Himmelfarb and Ikizler 2010). In essence, it acts as a long term kidney replacement by substituting for some of

the functions of the kidney and reducing the impact of the side-effects accompanying loss of kidney function. To accomplish this end, the patient's blood is pumped through a machine called a dialyser wherein solutes including urea, a toxic by-product of protein catabolism, are transported from the blood into the dialysate and solutes such as bicarbonate are transported from the dialysate into the blood (Figure 1.1) (Himmelfarb and Ikizler 2010).

Although hemodialysis has come a long way in the last several decades, due in large part to technical and technological advances, much research continues in the field. The usual treatment regime for in-center hemodialysis in North America is 4 hour sessions, 3 times a week. Increases in both time and frequency of treatments have shown improvements in solute and fluid removal (Himmelfarb and Ikizler 2010). Quality of life issues continue to be better addressed in part by the increased accessibility of in-home, rather than in-center, hemodialysis (Weinhandl et al. 2016).

Peritoneal dialysis is the second treatment option and gets its name from the use of the peritoneal cavity as the place of exchange for waste products out of the bloodstream. In peritoneal dialysis, rather than diverting the patient's blood out of the body into a dialyser, the dialysate is infused into the patient's abdomen via a permanent catheter. The dialysate remains in the abdomen for several hours during which time waste products diffuse across the peritoneum from the underlying blood vessels. Following the treatment period, the dialysate containing waste products is drained off and replaced with fresh dialysate (Figure 1.1). Although peritoneal dialysis offers many advantages

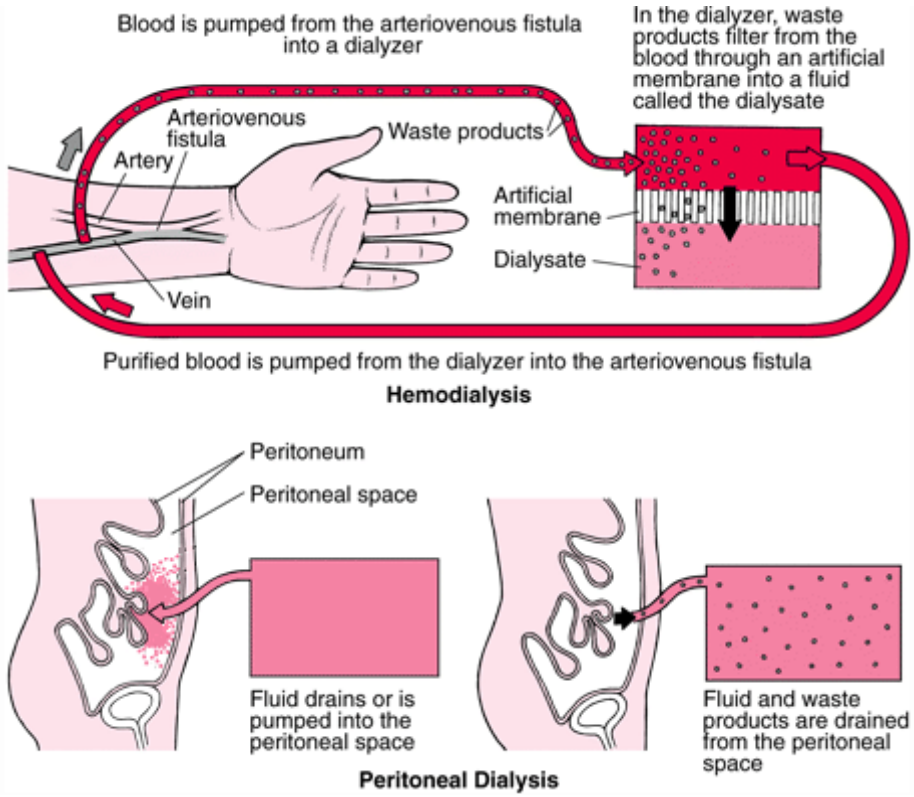

Figure 1.1. Comparison of dialysis modalities. In hemodialysis (top), blood is pumped through a dialyser where waste products are removed. In peritoneal dialysis (bottom), the dialysate is infused into the peritoneal cavity where it remains for several hours and is then drained along with waste products which diffuse from the underlying vasculature.

over hemodialysis, including in most cases lower associated costs (Bargman 2012), lesser requirement of highly trained personnel and infrastructure or equipment and general increase in freedom and improved quality of life of the patient, it remains underutilised in many parts of the world due in large part to socio-economic reasons (Abraham et al. 2015). Although peritoneal dialysis offers more flexibility and freedom to the patient in many regards, some patients also find the daily replacement of dialysate disruptive and disruption of the abdominal catheter can lead to peritonitis.

Finally, a transplanted kidney is considered by far the best option both financially and in terms of quality of life. A kidney from a living donor or a cadaver now has an average lifespan of 8-15 years depending on the type of graft (Thiruchelvam et al. 2011). More than 30% of living donors of kidneys are currently categorized as unrelated to their recipient. In order to avoid hyperacute rejection, protocols that desensitize and immunosuppress the recipient are now well established (Davis and Delmonico 2005). The main complications following transplant are rejection and infection, and maintenance immunosuppression is an ongoing requirement to prevent graft rejection. Although the transplant recipient will be closely followed and monitored for risk factors, including those of cardiovascular disease, the quality of life of a transplant patient is drastically improved. There are also many significant socioeconomic factors surrounding kidney transplants. Many countries in the world exploit their poor to supply kidneys for transplant. With a significant and maintained increase in "transplant tourism" and organ trafficking, participants in the International Summit on Transplant Tourism and Organ Trafficking held in Istanbul, Turkey in 2008 established The Declaration of Istanbul on Organ Trafficking and Transplant Tourism. Their hope being that "The legacy of transplantation must not be the impoverished victims of organ trafficking and transplant tourism but rather a celebration of the gift of health by one individual to another" (ISTTOT 2008).

There are also many groups working towards a wearable artificial kidney, which is not without its challenges. Conventional haemodialysis units usually weigh upwards of

60kg and also require a separate unit to purify the 120L of water that are required per session (Kooman et al. 2015). Finding ways to overcome the technical challenges associated with creating a device that is small enough to wear, functions adequately, is safe and will be adopted by the patients is not an easy task. Silicone nanopore membranes have recently been manufactured to serve as hemofilters for an implantable renal assist device (Kim et al. 2015). Other risks and challenges associated with a wearable kidney arise from the need for direct access of the device to the bloodstream, with the possibility of considerable blood loss or an air embolism in the case of an accidental disconnection (Kooman et al. 2015).

1.1.3 Stem Cell Based Therapies

Stem cells have been classically defined as the undifferentiated cells of a multicellular organism with extensive renewal capacity and the ability to generate daughter cells that undergo further differentiation (Watt and Driskell 2010). The general shift in interest towards stem cells is a focus on how adult tissue is maintained and repaired rather than how tissues arise during development in the embryo. Multiple studies have identified cell proliferation and some restoration of kidney function in animal models following ischemia. There is increasing evidence, although still debated, that podocyte and tubular progenitor cells exist that can to some extent replace damaged cells (Romagnani 2013, Romagnani et al. 2013, 2015). The controversial search for a renal stem cell continues and, to date, none have been identified that possess the indefinite capacity for self-renewal that in part defines a stem cell (Little and Bertram 2009, McCampbell and Wingert 2012, Endo et al. 2015). Various applications of embryonic stem cells, renal progenitor cells as well as induced pluripotent stem cells of various origin have been

pursued as a means to regenerate and repair kidney damage (Song et al. 2012, Yokote et al. 2012, Chou et al. 2014, Lam and Morizane 2015, Little and Takasato 2015, Suzuki et al. 2016).

Stem-cell based therapies which could allow for regeneration and preventative measures to kidney damage from disease will no doubt have far reaching implications and the potential to revolutionize the treatment of kidney disease (Yokoo et al. 2007, 2011). These strategies are still in their infancy and require a better understanding of kidney development at the cellular and molecular level, under both physiologic and pathologic conditions. Therapeutic targets will be identified as we continue to better understand how the same mechanisms involved in kidney development can be applied and targeted to repair kidney damage.

1.2 Kidney Development

Through the use of transgenic animals, much has been learned in the past decades about the molecular and cellular events that give rise to a fully functional kidney (For recent reviews see Costantini and Kopan 2010, Little et al. 2010, Rumballe et al. 2010, Little and McMahon 2012). However, it is clear that not all the players in the molecular pathways involved have been identified.

Kidney development results from the reciprocal signalling and interaction of the metanephric mesenchyme (MM) and the ureteric bud (UB) both of which are derived from the intermediate mesoderm (For a recent review see McMahon 2016). Briefly, at

embryonic day (E)10.5 in the mouse, factors secreted from the MM act on receptors in the UB and the UB subsequently invades the MM. At E11.5 the UB forms a T-shaped bifurcation and, in response to further signalling from the MM, will undergo several rounds of bifurcation and elongation which will ultimately result in the formation of the collecting duct system of the kidney (Dressler 2006). In parallel, signals from the UB induce the mesenchymal cells to condense around the ureteric bud tips as Six2 positive nephron progenitor cells (NPC) around the cap mesenchyme. These cells will subsequently proliferate and differentiate into pre-tubular aggregates which will, following mesenchymal-to-epithelial transition (MET), sequentially give rise to renal vesicles, comma-shaped and S-shaped bodies (S-SB) and finally functional nephrons (Kuure et al. 2000) (Figure 1.2). In the S-SB stage, the most distal portion of the S-SB unfolds and the lumen becomes continuous with the ureteric stalk, while the proximal cleft will be infiltrated by endothelial cells and form the glomerular tuft.

The functional subunit of the kidney is the nephron and each renal vesicle gives rise to a nephron. The renal corpuscle is composed of four primary cell types: endothelial cells, of the glomerular capillaries, intraglomerular mesangial cells, podocytes and parietal epithelial cells (Figure 1.3). The fenestrated endothelial cells of the capillaries form a primary component of the glomerular filtration barrier and allow proteins and fluid

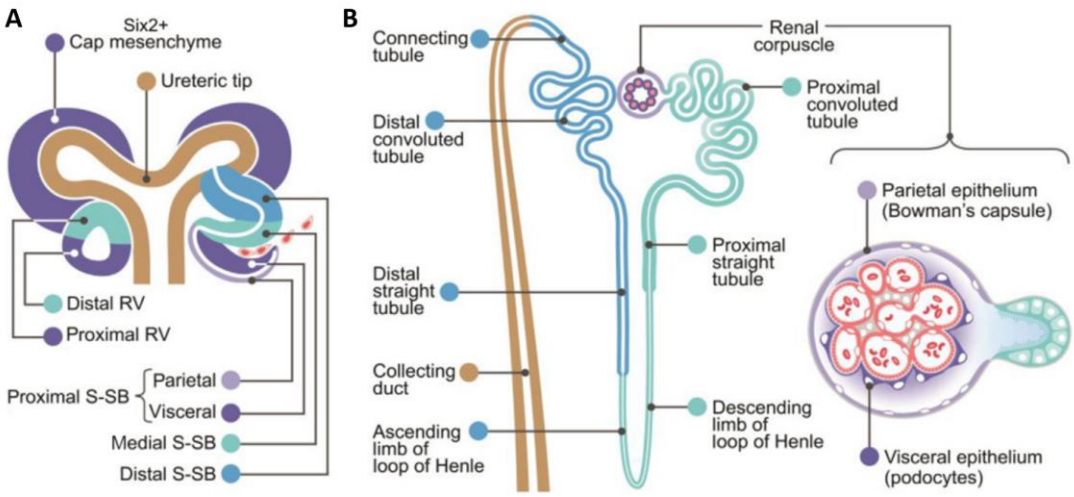

Figure 1.2. Schematic representation of the development of a nephron. A) Signaling from the ureteric bud tip causes the Six2+ cap mesenchyme to condense to form pretubular aggregates (not illustrated) which further differentiate and undergo mesenchymal-to-epithelial transition (MET) to form the renal vesicle (RV). The epithelial and polarized RV transitions through the comma-shape body (not illustrated) to the S-shape body (S-SB) to form the functioning nephron. B) The functional nephron. Colour coding indicates speculative cell lineage fate. Adapted from McMahon 2016.

to pass from the glomerular tuft to the urinary space. Mesangial cells provide physical support, elasticity and structure to the glomerular tuft. They also secrete extracellular matrix and act as pericytes to help regulate blood flow through the capillaries.

Podocytes are highly specialized epithelial cells that cover the glomerular capillaries, representing the visceral layer of Bowman's capsule. Sandwiched between the podocytes and the fenestrated endothelium of the capillaries is the glomerular basement membrane (GBM). A unique characteristic of the podocyte is the network of interdigitated foot processes and slit diaphragms that along with the GBM and underlying fenestrated endothelium make up the glomerular filtration barrier (Barisoni and Mundel 2003). As the ultrafiltrate travels through the glomerular filtration barrier it enters the urinary space of Bowman's capsule. The urinary (Bowman's) space is

surrounded by the parietal epithelium and feeds into the lumen of the proximal tubule.

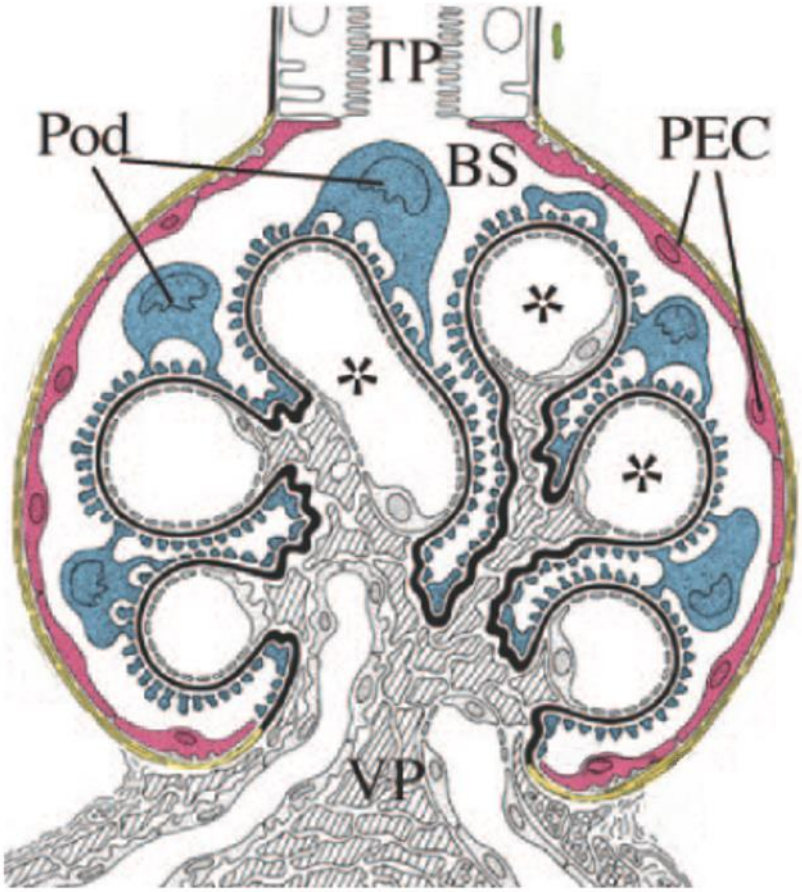

Figure 1.3. Schematic representation of the renal corpuscle. The blood enters the glomerular capillaries (*) from the vascular pole (VP). The fenestrated endothelium of the capillaries, glomerular basement membrane (black) and podocytes (Pod, blue) with slit diaphragms are the three components that make up the glomerular filtration barrier. The mesangial cells (shaded) provide physical support to the capillaries and also play a role in regulating blood flow. The parietal epithelial cells (PEC, red) encapsulate Bowman's space (BS) which is continuous at the tubular pole (TP) with the proximal tubule. Adapted from Appel et al. 2009.

Many of the specific molecular pathways involved in the maintenance of nephron progenitor cells, ureteric bud branching and proper nephron formation have been identified and continue to be elaborated.

Osr1 is one of the earliest markers of kidney progenitor cells in mice and has been shown to be upstream of other factors essential for normal kidney development including Eya1, Pax2, Six1 and Six2, WT-1 and Gdnf (James et al. 2006). Osr1 knockout mice fail to form intermediate mesoderm, resulting in renal agenesis. These animals die embryonically with cardiac defects (Wang et al. 2005). More recently Osr1 has been placed in a network of transcriptional regulators that mediate podocyte differentiation (Tomar et al. 2014) and shown to act synergistically with Wt1 to regulate nephron endowment (Xu et al. 2016).

Crucial to the proper formation of the outgrowth of the UB is the Gdnf/Ret pathway. Gdnf is secreted by the MM and acts on the Ret receptor-tyrosine kinase on the UB, which in turn triggers the outgrowth of cells towards the GDNF signal (Costantini 2010b). Many other pathways play critical roles in UB branching and MM differentiation, including bone morphogenic protein, sonic hedgehog, and fibroblast growth factors (Chai et al. 2013). Wnts have also been shown to play important roles in renal vesicle formation as well as in branching morphogenesis (Merkel et al. 2007).

Wnt9b and Wnt4 are known to play crucial roles in renal vesicle formation, while Wnt11 regulates UB branching by maintaining normal GDNF levels (Pepicelli et al. 1997, Merkel et al. 2007). Specifically signalling through β-catenin is essential for normal branching and maintenance of UB tip progenitor cell identity (Bridgewater and Rosenblum 2009, Little and McMahon 2012, Boivin et al. 2015). β-catenin has also been shown to play an essential role in the self-renewal of nephron progenitor cells (Karner et al. 2011, Sarin et al. 2014).

Advances in molecular biology techniques and approaches continue to increase our understanding of various genetic pathways in the kidney, in part by specifically targeting various cell types in the kidney (Kohan 2008, 2015). Defects in nephrogenesis and ureteric branching result in congenital anomalies of the kidney and urinary tract (CAKUT). These pleiotropic malformations comprise a multitude of renal phenotypes including renal agenesis, hypoplasia, and renal dysplasia, associated with obstructive or refluxive anomalies of the ureter (Toka et al. 2010). Congenital anomalies of the kidney and urinary tract are predicted to have different genetic origins (Weber 2012), yet the molecular pathogenesis of CAKUT is not well understood. Nephron deficiency is also a hallmark feature of CAKUT (Puddu et al. 2009). Our increased understanding of normal kidney development continues to set the stage for better appreciation of the pathogenesis of kidney disease and to allow for more comprehensive treatment strategies.

1.2.1 Nephron Number

An increasing body of evidence now links nephron endowment to long-term health. More recently, reduced nephron number has been hypothesized to constitute a "factor of vulnerability" which is insufficient by itself, however in combination with additional factors, can result in the early onset of renal diseases (Boubred et al. 2013). Early postnatal nutrition, including sodium intake as well as protein or caloric intake, along with early postnatal growth exert a considerable influence on adult health. Overfeeding and rapid postnatal growth both enhance the vulnerabilities acquired *in utero* and accelerate the development of adult diseases ("mismatch hypothesis") (Boubred et al. 2013). Almost three decades ago, Brenner et al. (1988) proposed that a decrease in the

total filtration surface resulting from reduced nephron number is associated with an adaptive increase in single nephron glomerular filtration rate (snGFR). This increase in snGFR in turn results in further adaptations including glomerular and tubular enlargement and elevated blood pressure levels (Brenner et al. 1988).

The principal determining factor associated with nephron number is birth weight but it is not the sole factor (Hughson et al. 2003). An increasing body of experimental evidence shows that low birth weight and reduced nephron number can occur independent of each other and do not always result in adult hypertension (Mitchell et al. 2004). It has also been shown that maternal dietary protein restriction leads to reduced glomerular number, impairment of renal development and programs for salt-sensitive adult hypertension in rodents (Woods et al. 2004). Using a mouse model, Walker et al. (2012) showed that increased nephron endowment protects against salt-induced hypertension. Other factors of significant influence include the timing and nature of *in utero* insults, such as excessive glucocorticoid exposure or maternal malnutrition, the gender of the fetus and the postnatal environment (Moritz et al. 2003, Moritz and Bertram 2006, Benz and Amann 2010). Variability in nephron endowment may be explained in part, by genetic and environmental factors as well as the interaction between the two.

Glomerular volume varies inversely with glomerular number. Larger glomeruli may be a sign of compensatory hyperfiltration and hypertrophy in individuals with fewer nephrons. Based on this observation, it has been suggested that when kidney damage occurs, the total filtration surface area may initially be maintained. However, the accompanying glomerular hypertrophy is a predictor of poorer outcomes

(Douglas-Denton et al. 2006, Zandi-Nejad et al. 2006), such as systemic hypertension, albuminuria, and reduced glomerular filtration rate (GFR) culminating in ESKD (Schreuder 2012).

In order to quantify nephron number, multiple approaches have been developed, the most accurate and unbiased being the application of stereology. The application of stereology, both technical and theoretical, to kidney research has been covered at length elsewhere (Nyengaard 1999, Cullen-McEwen et al. 2012a, 2012b, Puelles et al. 2014, Puelles and Bertram 2015). Stereological techniques can be used not only for counting nephrons and other cell types found in the kidney, but can also be applied to make unbiased volumetric measurements. Tissue shrinkage as well as other deformations, including expansion of the paraffin embedded section when floated on a hot water bath, make it impossible to obtain unbiased and accurate measurements of volume without the use of plastic polymers as embedding medium and utmost consistency in technical protocols.

Much still remains to be discovered in our understanding of the complex relationship between nephron endowment, hypertension and kidney disease. Various studies have shown that reduced nephron number, especially when it is moderate, is not systematically associated with impaired GFR and hypertension (Zimanyi et al. 2004, Hughson et al. 2006, Boubred et al. 2009). Many studies suggest that the relationship between nephron number, hypertension and kidney disease is quite complex and multifaceted. The clinical outcome depending not only on the severity of reduced nephron endowment, but also the degree of increased snGFR (Moritz et al. 2009,

Boubred et al. 2013). Multiple genes have been identified that are linked to an impairment of nephron development in humans. Some are associated with a reduction in the number of nephrons and kidney size, such as *PAX2*, which is associated with renal coloboma, while others, such as *EYA1*, are associated with more severe renal agenesis (Dötsch et al. 2009).

1.3 Sox Genes

Sry-related high-mobility group (HMG) Box (Sox) genes are a family of transcription factors characterized by the HMG domain, which is a DNA-binding domain shown to interact with the minor groove of the DNA helix. Although often studied in isolation, the binding of transcription factors to their regulatory sequences are but a single event in a series of complex interactions that ultimately regulate gene expression (Spitz and Furlong 2012, Huang et al. 2015). The resolved crystal structures of various HMG domains from the Sox family of transcription factors has increased our understanding of the conformations that allow for preferential binding to DNA sequences (Jauch et al. 2012). All Sox proteins recognize and bind the DNA consensus motif A/TA/TCAAA/T. This sequence is short and degenerate and found in abundance throughout the genome. Although flanking sequence preferences have also been identified for many of the SOX proteins, such that the position weighted consensus sequence for SOX4 binding is AACAAA/TG/AG/A (Scharer et al. 2009), other factors must be involved in assigning specificity of recognition for a given SOX protein to the appropriate sequence. The activity of Sox proteins is essentially dependant on their DNA-binding properties, however their ability to bind to the appropriate DNA sequences is also dependant on their interactions with other transcription factors and co-factors (Wegner 2010).

Sox genes are differentially expressed over time and in various compartments throughout development of multiple organ systems (Wegner 1999, Watanabe et al. 2016). Mammalian Sox genes are divided into eight subfamilies (A-H) based on phylogenetic homology of their HMG box domain (Kiefer 2007). The Sox family of transcription factors have been shown to control stem cell self-renewal and stem cell differentiation in a multitude of developmental processes (Sarkar and Hochedlinger 2013), including stemness (Avilion et al. 2003), male differentiation (Koopman et al. 1991, Foster and Graves 1994, Schepers et al. 2003, Chaboissier et al. 2004, Koopman 2005), neurogenesis (Graham et al. 2003, Episkopou 2005) and gliogenesis (Kuhlbrodt et al. 1998a, 1998b), neural crest (Hong and Saint-Jeannet 2005) and spinal cord development (Shim et al. 2012), cardiac outflow tract (Schilham et al. 1996) and B-cell development (Wetering et al. 1993), as well as skeletogenesis (Zhao et al. 1997, Lefebvre et al. 1998, Lefebvre and Smits 2005, Lefebvre and Bhattaram 2016) *in vivo*.

Members of a given Sox subfamily are often found co-expressed with each other in developing tissues and have been shown to exhibit overlapping functions. Accordingly, the three SoxC subfamily members *Sox4*, *Sox11* and *Sox12* exhibit overlapping expression patterns in many developing tissues during embryonic development (Dy et al. 2008), and essential overlapping functions of *Sox4* and *Sox11* have been demonstrated in neural progenitor cell survival and differentiation during organogenesis *in vivo* (Thein et al. 2010, Shim et al. 2012, Mu et al. 2012). *Sox4* has also been identified to play multiple roles in and is correlated with a variety of tumours (Jafarnejad et al. 2013, Vervoort et al. 2013), including leukemia (Zhang et al. 2013),

pancreatic cancer (Huang et al. 2012), lung cancer (Castillo et al. 2012), breast cancer (Tiwari et al. 2013) and colorectal cancer (Andersen et al. 2009), while *Sox11* has been identified in association with mantle cell lymphoma (Lu et al. 2013) as well as gastric cancer (Qu et al. 2014).

Sox4 knockout mice die at E14 from a defect in cardiac outflow tract formation (Schilham et al. 1996), while *Sox11* knockout mice die shortly after birth with a similar, although less severe, malformation of the heart (Sock et al. 2004). *Sox12* knockout mice on the other hand are viable and fertile and do not appear to have any defects (Hoser et al. 2008). It has been shown, by evaluating combinations of heterozygous, double- and triple-knockout mice, that the three SoxC genes interact functionally with each other during organogenesis and that functional redundancy exists between the three SoxC genes *in vivo* in a multitude of developmental processes (Bhattaram et al. 2010). Of the three SoxC genes, SOX12 has been shown to be the weakest transactivator *in vitro* (Hoser et al. 2008), and most recent studies of SoxC gene function have focused only on *Sox4* and *Sox11*.

1.3.1 Sox Genes in the Kidney

Multiple studies have reported on the presence of a *Sox* gene being expressed in the kidney without further follow-up or investigation. Takash et al. (2001) detected mRNA expression of *Sox7* and *Sox18* in the kidney by Northern Blot. The signal was not very strong compared to other tissues or embryonic stages but was nonetheless present (Takash et al. 2001). Moderate levels of *Sox18* expression were also detected in the kidney by RT-PCR by Hosking et al. (2001). By whole mount *in situ* hybridization,

Sox8 was shown to be expressed in the kidney at E13.5 at the tips of the ureteric trees (Schepers et al. 2000) and multiple groups have also identified the expression of *Sox13* in the kidney (Kido et al. 1998, Roose et al. 1998, Kasimiotis et al. 2000).

Still other studies have linked the expression of Sox genes to the development of diabetic nephropathy and other defects in kidney development. *Sox17* expression was found to be decreased in the early phase of diabetic nephropathy in a murine model (Wada et al. 2001) and mutations in *Sox17* are also associated with CAKUT in humans (Gimelli et al. 2010, Combes et al. 2012). Matsui et al. (2006) provided evidence of functional redundancy of *Sox17* and *Sox18* in vascular endothelial cells by generating double mutant mice. Specifically they demonstrated that although $Sox18^{-/-}$ and $Sox17^{+/-}$; $Sox18^{+/-}$ double transgenic mice presented no vascular defects, by removing an additional allele approximately half of the $Sox17^{+/-}$; $Sox18^{-/-}$ mice die before postnatal day (P)21 due to reduced neovascularization of the liver and kidney as a result of defective endothelial remodeling (Matsui et al. 2006). These experiments demonstrate that in the total absence of *Sox18*, *Sox17* is able to fill in for *Sox18* with no apparent changes in the phenotype.

Sox8, *Sox9*, and *Sox10* make up the *SoxE* subfamily of transcription factors. The *SoxE* subfamily has been shown to play multiple roles in various cell types during kidney development. *Sox9* plays a role in the regulation of α2(IV) collagen in mesangial cells (Sumi et al. 2007), and as a result of signaling, also induces a chondrogenic phenotype of mesangial cells during diabetic nephropathy (Kishi et al. 2011). Recently two groups have shown that *Sox9* activation is an early transcriptional response to acute kidney

injury which is required for normal proximal tubular repair after acute ischemic kidney injury. *Sox9* positive progenitor cells also contribute to epithelial regeneration following injury (Kumar et al. 2015, Kang et al. 2016).

In 2001 Sock et al. generated *Sox8* knockout mice by replacing the endogenous *Sox8* by the *LacZ* reporter gene and a *neo* cassette. The *LacZ* gene codes for the β-galactosidase enzyme and its expressions allows for the easy identification of cells where it is expressed through incubation with the X-Gal substrate resulting in a blue precipitate. This replacement allowed not only inhibition of the expression of *Sox8*, but also use of the LacZ marker to identify the location of endogenous *Sox8* (Sock et al. 2001). The resulting mice were viable, fertile, and had a substantial weight reduction, however they failed to exhibit any major developmental defects in any of the organs where *Sox8* is expressed. Although there was no compensatory increase in *Sox9* or *Sox10* expression, the mild phenotype in the absence of *Sox8* could at least in part be due to functional redundancy between the three proteins. With the expression LacZ in various organs, including the kidney, *Sox8* was identified and tracked over time. Expression of *Sox8* in the kidney starts at the tip of the ureteric tree and from E14.5 onward it is restricted to the glomeruli and proximal tubules. At the conclusion of nephrogenesis, the expression of *Sox8* rapidly decreases and is not detectable in the adult kidney (Sock et al. 2001). More recently, *Sox8* and *Sox9* have been shown to play an important role in ureteric branching during kidney development. Using two conditional knockouts strains, Airik et al. (2010) demonstrated that the absence of *Sox9* expression in the undifferentiated ureteric mesenchyme resulted in hydroureter due to a functional obstruction. There was no effect on condensation, proliferation and apoptosis of the undifferentiated

mesenchyme (Airik et al. 2010). *Sox9* acts at multiple steps during kidney development, and significantly acts with *Sox8* to regulate epithelial branching morphogenesis of the ureter. *Sox9* also maintains ureteric tip identity. In the absence of *Sox9* ectopic nephron formation occurs in the outer cortex of the kidney (Reginensi et al. 2011). These data taken together indicate a significant role for the *SoxE* subfamily of transcription factors in kidney development and maintenance.

Prior to the research presented in this thesis, very little has been known of the role of *SoxC* in kidney development. SOX11 and WT1 proteins have been immunoprecipitated together in protein extracts from embryonic mouse kidneys using an anti-SOX11 antibody, suggesting that they interact *in vivo* (Murugan et al. 2012). Morpholino mediated knockdown of *wt1* or *sox11* also inhibited the expression of the epithelialization factor *wnt4* in the pronephros of *Xenopus* embryos (Murugan et al. 2012). In 2000, Plisov et al. published the results of a differential display experiment in which they induced rat MM explants to either condense, or to form tubules. In this system, *Sox11* was identified as one of the novel sequences found to be increased during MET in the kidney. They also confirmed, with *in situ* hybridization, the findings of their differential display and found that *Sox11* was most markedly expressed in the newly formed epithelial structures of the S- and comma-shaped bodies (Plisov et al. 2000). Although Murugan et al. (2012) were able to show physical interaction of WT1 and SOX11 proteins in a complex in the developing murine kidney at E13.5, there has been no direct evidence presented as to the role that *Sox11* plays in the mammalian kidney. As noted above MET plays an important roles during normal kidney development, and it is interesting to note that *Sox11* is upregulated during epithelial-to-mesenchymal transition (Kiemer et al. 2001, Venkov et

al. 2011) and that it has been identified by differential display in induced rat kidney explants during MET (Plisov et al. 2000). Further all three members of the *SoxC* subfamily (*Sox4*, *Sox11*, and *Sox12)* were identified as candidate Wt1 gene targets by ChIP-chip in E18.5 mouse kidneys (Hartwig et al. 2010). Taken together, this data suggests that the expression of *SoxC* in the mammalian kidney merits further investigation.

1.4 Research Objective

The purpose of this dissertation is to establish the role of *Sox4* in kidney development with a particular focus on nephron progenitor cells. All experiments were carried out to test the working hypothesis that *Sox4* is required for normal murine kidney development *in vivo.* I first sought to establish the expression profile of *Sox4* during murine kidney development. As *Sox4* knockout mice die embryonically prior to the completion of nephrogenesis, conditional knockout mouse models were further used to elucidate the role of *Sox4* in nephron progenitor cells and podocytes.

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2. The Transcription Factor Sry-Related HMG Box-4 (SOX4) is Required for Normal Renal Development *in vivo*

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2.1 Abstract

The DNA-binding transcription factor Wilms' Tumor Suppressor-1 (WT1) plays an essential role in nephron progenitor cell differentiation during renal development. Previously, chromatin-immunoprecipitation coupled to microarray (ChIP-chip) was used to identify novel WT1 target genes that may regulate nephrogenesis *in vivo*. It was discovered that all three members of the *SoxC* subfamily, namely, *Sox4*, *Sox11* and *Sox12,* are bound by WT1in mouse embryonic kidneys *in vivo. SoxC* genes play master roles in determining neuronal and mesenchymal progenitor cell fate in a multitude of developmental processes, but their function in the developing kidney is largely unknown. Results from the present study show that all three *SoxC* genes are expressed in the nephrogenic lineages during renal development. Conditional ablation of *Sox4* in nephron progenitors and their cellular descendants (*Sox4*$^{neprhon-}$ mice) results in a significant reduction in nephron endowment. By postnatal day (P)7, *Sox4*$^{neprhon-}$ renal corpuscles exhibit reduced numbers of WT1+ podocytes together with loss of expression of the slit diaphragm protein nephrin. *Sox4*$^{neprhon-}$ mice develop early-onset proteinaceous glomerular injury within 2 weeks of birth progressing to end-stage renal failure within 5-9 months. Collectively, these results demonstrate an essential requirement of *Sox4* for normal renal development *in vivo.*

2.2 Introduction

Kidney development begins at embryonic day (E)10.5 in mice, with the outgrowth of the ureteric bud from the Wolffian duct into the overlying metanephric mesenchyme (for a recent review, see Little and McMahon 2012). In response to inducing signals from the ureteric bud, the metanephric mesenchyme condenses as a tight cap of nephron progenitor cells around ureteric bud tips, while the ureteric bud branches to form the collecting system. During nephrogenesis, nephron progenitors have the capacity to self-renew and to differentiate into pretubular aggregates, which in turn undergo mesenchymal-epithelial transition to sequentially form early renal vesicles, comma-shaped bodies, S-shaped bodies, and, ultimately, functional nephrons (Saxén and Sariola 1987, Self et al. 2006, Kobayashi et al. 2008).

Defects in nephrogenesis and ureteric branching result in congenital anomalies of the kidney and urinary tract (CAKUT). With an incidence of 1:400 (Pope et al. 1999, Rumballe et al. 2010), CAKUT constitute one of the most frequent birth defects in humans, and the major cause of childhood renal failure. These pleiotropic malformations comprise a multitude of renal phenotypes including renal agenesis, hypoplasia, and renal dysplasia, associated with obstructive or refluxive anomalies of the ureter (Toka et al. 2010). CAKUT are predicted to have different genetic origins (Weber 2012), yet the molecular pathogenesis of CAKUT is not well understood. Nephron deficiency is a hallmark feature of CAKUT (Puddu et al. 2009). In fact, nephron number varies greatly even within normal range, from 7,000 to 20,000 in mice (Cebrian et al. 2004, Murawski et al. 2010, Zhong et al. 2012), and from 200,000 to 2 million in humans (Hughson et al.

2003, Hoppe et al. 2007, Bertram et al. 2011). Low nephron endowment within this normal range, though asymptomatic early in life, is associated with adult-onset hypertension (Mackenzie and Brenner 1995, Walker et al. 2012), a leading cause of coronary heart disease, stroke, and renal failure in North America (Tedla et al. 2011). Despite the fundamental importance of nephron endowment to health and disease, only a minority of molecular mechanisms underlying nephron morphogenesis have been identified.

The *Wilms' Tumour Suppressor-1*(*WT1*) gene encodes a DNA- and RNA-binding nuclear transcription factor that plays a critical role during nephrogenesis in part via its target gene effectors. However, endogenous gene targets that mediate WT1 function during renal development are largely unknown. Previously, WT1 ChIP-chip genome-wide location analysis was employed in embryonic E18.5 mouse kidney tissue, as a means of identifying novel kidney development genes that might act down-stream of Wt1 to control nephrogenesis *in vivo* (Hartwig et al. 2010). Intriguingly, all three members of the *Sry-related high-mobility group (HMG) Box (Sox)-C* subfamily—*Sox4*, *Sox11*, and *Sox12*—were identified as candidate WT1 gene targets by ChIP-chip. Members of the Sox gene family have been shown to play master regulatory roles in a multitude of developmental processes including dorso-ventral patterning (Chen et al. 2012), stemness (Avilion et al. 2003, Masui et al. 2007, Wang et al. 2012), male differentiation (Koopman et al. 1991, Wagner et al. 1994), neurogenesis (Bylund et al. 2003, Taranova et al. 2006, Bhattaram et al. 2010, Bergsland et al. 2011, Mu et al. 2012), cardiac outflow tract, B-lymphocyte (Schilham et al. 1996), and spinal cord development (Shim et al. 2012), and skeletogenesis (Akiyama and Lefebvre 2011, Lefebvre and Bhattaram 2016).

However, SOX function in the developing kidney is largely undefined.

Mammalian Sox genes are categorized into eight subgroups (A–H) based on phylogenetic homology of their HMG box DNA-binding domain. SOX proteins from different subgroups share partial identity (<46%) in their DNA-binding domain and none outside this domain. In contrast, subgroup members share a high degree of identity both within and outside their DNA-binding domains, thus often exhibiting functional redundancy in tissues where they are co-expressed. Accordingly, all three members of the *SoxC* subfamily— *Sox4*, *Sox11*, and *Sox12*—exhibit overlapping expression patterns in multiple tissues during embryonic development, suggesting genetic redundancy within this subfamily (Dy et al. 2008, Hoser et al. 2008). In this study, the renal developmental expression pattern of the three *SoxC* genes is characterized, and shown that strong *Sox4* expression is detected in nephron progenitors and their derivative nephrogenic structures, while *Sox11* appears preferentially expressed in differentiating nephrogenic structures during renal development. To begin the investigation of *SoxC* function during nephrogenesis, *Sox4* function was ablated in nephron progenitors and their cellular descendants ($Sox4^{nephron-}$ mice) using an established *Six2Cre-EGFP* transgenic line (Kobayashi et al. 2005, 2008). Postnatal $Sox4^{nephron-}$ kidneys exhibit significantly reduced nephron number. $Sox4^{nephron-}$ mice exhibit reduced podocyte nephrin expression at P7, and develop severe proteinaceous kidney injury within 2 weeks of birth, leading to end-stage renal disease. Collectively, these results demonstrate an essential requirement for Sox4 in normal renal development, and further support a role for *Sox4* in glomerular development and/or adult glomerular maintenance *in vivo*.

2.3 Materials and Methods

2.3.1 Histology, Immunofluorescence, and RNA *In Situ* Hybridization

For histology, postnatal (P) kidney tissues (P7 - *Sox4*$^{\text{nephron-}}$ n=4, wild-type n=4; P16 - *Sox4*$^{\text{nephron-}}$ n=7, wild-type n=7; Adult - *Sox4*$^{\text{nephron-}}$ n=19, wild-type n=12) from male mice were fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO), and processed to form paraffin blocks in a Tissue-Tek VIP 6-A1 vacuum infiltration processor (Sakura Finetek, Torrance, CA) . The following program was used for tissue processing: Formalin 60 seconds, 70% EtOH 60 seconds, 70% EtOH 60 seconds, 95% EtOH 60 seconds, 95% EtOH 60 seconds, 95% EtOH 60 seconds, 100% EtOH 60 seconds, 100% EtOH 60 seconds, xylene 90 seconds, xylene 90 seconds, paraffin 30 seconds, paraffin 30 seconds, paraffin 30 seconds, paraffin 30 seconds. Tissues were sectioned at a thickness of 5 µm and subsequently stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). For indirect immunofluorescence, embryonic kidneys (*Sox4*$^{\text{nephron-}}$ n=3, wild-type n=3) and postnatal (*Sox4*$^{\text{nephron-}}$, n=3, wild-type n=3) kidneys were frozen in Tissue-TEK 4583 OCT compound (Sakura Finetek) in a liquid nitrogen cooled bath. To visualize nephrin expression, the kidneys were fixed in 4% paraformaldehyde (Sigma-Aldrich) at 4°C overnight before embedding in OCT compound. For all other antibodies, cryosections were fixed in methanol (Sigma-Aldrich) at -20°C for 10 minutes. Immunofluorescence staining on 4 midline 4-µm cryosections was performed as described (Hartwig et al. 2010). The primary antibodies used were as follow: rabbit anti-Wt1 (C-19, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-E-Cadherin (610181, BD Bioscience, San Jose, CA), goat anti-nephrin (N-20, Santa Cruz), rat anti-β1 integrin (MAB1997, Chemicon, Temecula, CA), rabbit anti-podocin (P0372, Sigma, St. Louis, MO), rabbit anti-NG2 chondroitin

sulfate (AB5320, Millipore, Billerica, MA), rabbit anti-PDGFRb (3169, Cell Signaling, Danvers, MA), rat anti- nidogen (MAB 1883, Chemicon), rat anti-PECAM1 (553370, BD Biosciences), and mouse anti-desmin (M0760, Dako, Carpinteria, CA). Fluorescence was detected using secondary antibodies conjugated to Texas Red and fluorescein isothiocyanate fluorochromes (Jackson ImmunoResearch, West Grove, PA). RNA *in situ* hybridization was performed on embryonic and postnatal frozen kidney sections at a thickness of 8 μm using antisense probes. Corresponding sense negative controls did not produce background hybridization signals. *SoxC* probes were generated by PCR amplification using the following primers: *Sox4* forward, 5'GAACGCCTTTATGGTGTGGT3'; *Sox4* reverse, 5'GGTAGACGCGCTTCACTTTC3'; *Sox11* forward, 5'GAGCCTGTACGACGAAGTGC3'; *Sox11* reverse, 5'TCAAAGAGCCACAAGCTTCA3'; *Sox12* forward, 5'AGAGCGGGTTCTCTTTAGGC3'; *Sox12* reverse, 5'TCAGCATGGGACAACACATT3'. PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA).

2.3.2 Glomerular Quantification

Glomeruli were counted as described using the acid maceration method (MacKay et al. 1987, Godley et al. 1996, Hoy et al. 2011). Briefly, the left kidney of each animal was decapsulated (*Sox4*$^{\text{nephron-}}$ n=4, wild-type n=4), cut into 2 mm^3 pieces and incubated in 5 mL of 6 N HCL at 37°C for 90 min, and tissue fragments were gently disrupted by aspirating up and down using a 10 mL pipette. The suspension was diluted to a total volume of 30 mL with water and incubated at 4°C overnight with rotation. The

preparation was resuspended and a 500 mL aliquot removed to a counting chamber. Glomerular number was counted under phase microscopy in triplicate. The number of glomeruli per kidney was determined by correcting the mean of the glomerular counts for dilution.

2.3.3 Reverse Transcription Quantitative PCR (RT-qPCR)

For embryonic stages, mouse embryos were excised from pregnant CD1 mice. Total RNA was isolated from 3 separate pooled biological samples from 9 to 10 pairs of kidneys at E11.5, E12.5, 5 to 6 pairs of kidneys at E14.5, and 2 to 3 pairs of kidneys at E16.5 A single kidney was collected at E18.5, P7 and P21 from 3 separate animals. Freshly harvested mouse kidneys were stabilized in RNA Later (Ambion, Austin, TX) at 4°C overnight with rotation and then stored at -80°C according to manufacturer's instructions. Tissues were homogenized using a Tissue-Tearor (Biospecifics Products, Lynbrook, NY) and total RNA was extracted using the RNAqueous 4-PCR kit (Ambion). RNA was reverse-transcribed using the Superscript III First-Strand cDNA Synthesis System (Invitrogen). For quantification of SoxC mRNA levels, real-time PCR was performed using PerfeCTa SYBR Green Master Mix (Quanta Biosciences, Gaithers-burg, MD) and the Rotor-Gene 3000 detection system (Qiagen, Valencia, CA). The following SoxC primer sequences were used: *Sox4* forward, 5'GATCTCCAAGCGGCTAGGCAAA3'; *Sox4* reverse, 5'GTAGTCAGCCATGTGCTTGAGG3'; *Sox11* forward, 5'GGACCTGGATTCCTTCAGTGAG3'; *Sox11* reverse, 5'GTGAACACCAGGTCGGAGAAGT3'; *Sox12* forward, 5'CATTTCGAATTCCCGGACTA3'; *Sox12* reverse,

5’GGTCGGCGATACTAGACGAG 3’.

For absolute copy number determination, standard plots were constructed from known concentrations of serially diluted full-length SoxC cDNA plasmids (TrueORF Gold cDNA in pCMV6-Entry, OriGene Technolo2gies, Rockville, MD) over the concentration range of 10^1-10^7 copies/mL, as previously described (Pfaffl and Jolla 2004, Dhanasekaran et al. 2010). Each standard dilution point was performed in triplicate over the complete standard-curve range to achieve a reliable standard curve for each measured parameter ($R^2 > 0.99$; Efficiency (E) ≥ 1.95). Standard curves were performed for both target and plasmid cDNA in 3 or more replicates on different days to validate reproducibility (in all cases, $R^2 > 0.99$, $E \geq 1.95$). qPCR experiments and analyses were performed with consideration for the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2010). Appendix C contains a table outlining relevant MIQE technical information.

2.3.4 Mouse Strains

The Sox4 null (*Sox4*$^-$) allele (Schilham et al. 1996), conditional Sox4 (*Sox4*^{fx}) allele (Penzo-Méndez et al. 2007) and Six2-Cre BAC transgenic line (Kobayashi et al. 2008) have been previously characterized. All animal experiments were carried out in accordance with the policies of the Animal Care Committee at the Atlantic Veterinary College, University of Prince Edward Island.

2.3.5 Transmission Electron Microscopy

Kidneys were cut into 3–4 mm thick sections, immediately immersed in 2% glutaraldehyde (SP1 Supplies, West Chester, PA) in 0.1 M phosphate buffer, pH 7.2,

and refrigerated for 30 min at 4°C. The cortical region was trimmed from each section and cut into smaller pieces and stored in 2% glutaraldehyde in phosphate buffer at 4°C overnight. Tissues were washed twice with phosphate buffer, post-fixed in 1% osmium tetroxide (SP1 Supplies) in phosphate buffer pH 7.2, for 1 hr at room temperature, then washed for 10 min in 0.1 M phosphate buffer. Tissue was dehydrated through a graded series of ethanols, cleared in propylene oxide (SP1 Supplies), infiltrated and embedded in Epon (SP1 Supplies). Semi-thin sections (0.5 mm) were cut and stained with 1% toluidine blue in 1% sodium tetraborate solution and examined with a light microscope. All samples that contained at least two glomeruli were re-cut to generate ultrathin sections (90 nm). Ultrathin sections were stained with uranyl acetate and Sato's lead stain. Sections were examined using a Hitachi H7500 transmission electron microscope operated at 80 kV and digital images were captured using an AMT XR40 side-mounted camera.

2.3.6 Statistical Methods

Two sample t-tests were performed using Minitab 16. Data are presented as mean ± standard error.

2.4 Results and Discussion

2.4.1 Dynamic Distribution of SoxC Transcripts During Renal Development

Quantitative real-time qPCR and RNA *in situ* hybridization was performed to analyze the expression of *SoxC* transcripts at multiple stages of embryonic renal development, from the onset of renal development at E11.5 until E18.5, as well as at two post-natal

time-points, namely, P7 when nephrogenesis is completed, and in adult P21 kidneys (Figure 2.1). Absolute quantitative qPCR (normalized to 25 ng RNA) shows that the three *SoxC* transcripts are highly expressed throughout renal development and in all cases are expressed at the lowest level in the adult (Figure 2.1A–C). Reference genes evaluated for qPCR using all three biological replicates for each time point were *Gapdh*, *Pgk1* and *Hprt1*. *Pgk1* was determined to be the most stable reference gene using both the NormFinder (Andersen et al. 2004) and BestKeeper (Pfaffl et al. 2004) algorithms. Trends of *SoxC* absolute copy number expression are maintained when normalized to *Pgk1* (Appendix A). Preliminary evaluation of post-natal time points from P0 to P14 shows that relative expression of *Sox4* remains high from P0 to P9 before being reduced to the lowest level of expression in the adult (Appendix B). Further post-natal data will be needed to confirm this observation.

Similar to previous reports of overlapping *SoxC* expression in developing tissues (Dy et al. 2008), *SoxC* transcripts exhibit an overlapping but distinct pattern of expression in the early developing kidney: *Sox4*, *Sox11,* and *Sox12* are all expressed in nephron progenitors and in the ureteric bud (Figure 2.1D–F). Notably, strong *Sox11* expression is also detected in epithelialized nephrogenic structures at E12.5. As development proceeds, strong *Sox4* and *Sox12* expression continues to be detected in nephron progenitors and ureteric bud lineages as well as in differentiating nephrogenic structures, as shown at E18.5 (Figure 2.1G,G',I,I'). Similarly, *Sox11* expression continues to be strongly detected in differentiating nephrogenic structures throughout renal development, including pretubular aggregates, comma-shaped and S-shaped

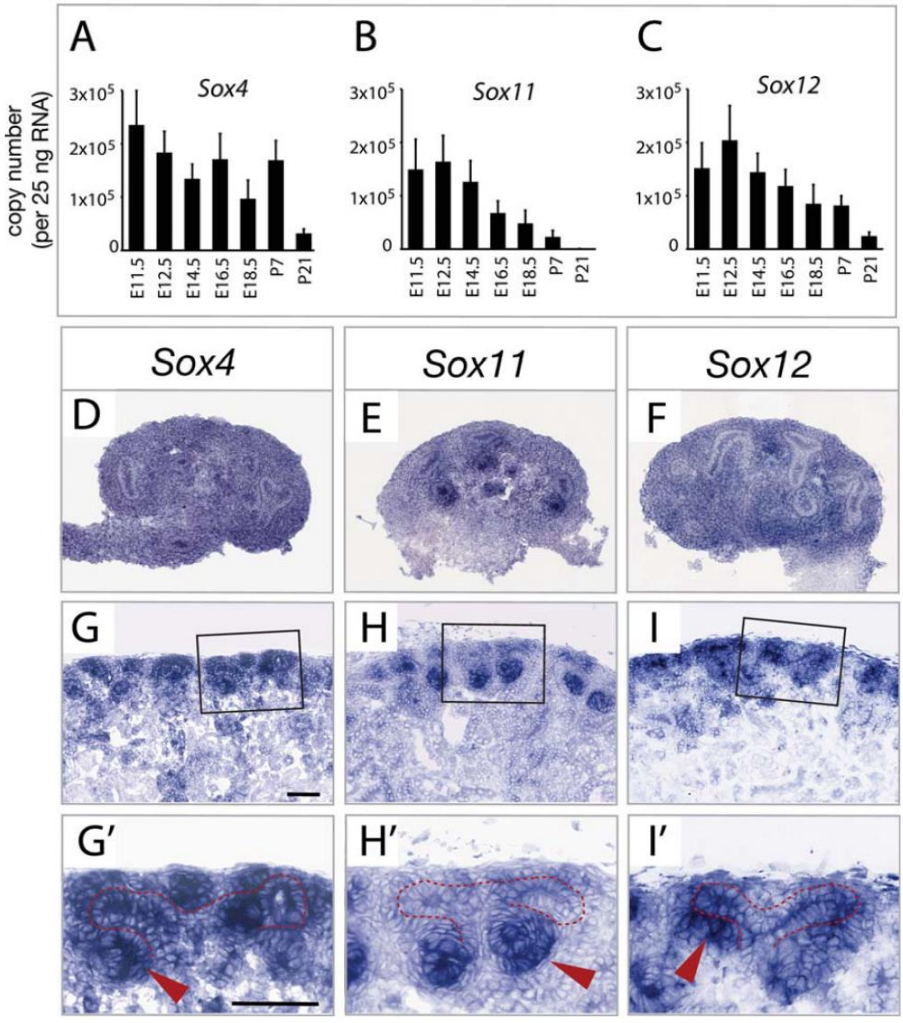

Figure 2.1. Expression of *SoxC* transcripts in the developing mouse kidney. A–C: Absolute copy number of *SoxC* transcripts (normalized to 25 ng total RNA) during renal development was detected by reverse transcription quantitative PCR (RT-qPCR) with specific primers recognizing *Sox4*, *Sox11*, and *Sox12*, using calibration curves from serially diluted recombinant SoxC plasmid DNA (n = 3 biological replicates). RNA from embryonic kidneys was dissected at E11.5, E12.5, E14.5, E16.5, E18.5, as well as at P7 and P21. Data are Mean + SE. D–F: *Sox4* and *Sox12* are strongly expressed in nephron progenitors and in the ureteric bud lineages during early renal development as shown at E12.5, while *Sox11* is preferentially expressed in pretubular aggregates. G–I, G'–I': *Sox4* and *Sox12* continue to be expressed in nephron progenitors and ureteric bud during late renal development (E18.5), as well as in epithelialized nephrogenic structures together with *Sox11* (arrowheads). Dashed red - outline of ureteric bud. Scale bar = 100 μm.

bodies, with weak expression in nephron progenitors and ureteric bud (Figure 2.1H,H').

Nephrogenesis is complete within the first week of postnatal life, coincident with the absence of expression of nephron progenitor markers including *Six2, Cited1, Meox1,* and *Dpf3* (Mugford et al. 2009, Brown et al. 2011). At this stage, diffuse *Sox4* and *Sox12* expression are detected in multiple cell types of the kidney, and low levels of *Sox11* are also detected. Focal *SoxC* expression is observed in epithelialized tubules primarily in the renal papilla, and declines to low levels by P21 (data not shown). Consistent with the established roles for *Sox4* and *Sox11* during embryonic development, these expression data suggest a primary role for *SoxC* genes in directing different stages of nephrogenesis during renal development *in vivo*.

SoxC proteins share a high degree of identity in their high-mobility group domain, and in the SoxC-specific transactivation domain. Of the three *SoxC* genes, *Sox12* is the weakest transactivator in vitro (Dy et al. 2008). $Sox12^{-/-}$ mice develop normally, are fertile, and do not exhibit any obvious defects (Hoser et al. 2008). Genetic analyses of embryos with all combinations of *SoxC* -null alleles reveals that loss of *Sox12* only slightly aggravates the phenotype of $Sox4^{+/-};Sox11^{+/-}$ and $Sox4^{-/-}; Sox11^{-/-}$ embryos (Bhattaram et al. 2010). Collectively, these genetic studies demonstrate that *Sox12* is dispensable, providing only minor contributions during embryonic development *in vivo*, possibly due to low transcriptional competence of its protein product.

In contrast, independent, essential roles for *Sox4* and *Sox11* have been demonstrated in heart development *in vivo*. $Sox4^{-/-}$ mice die prior to E14 due to cardiac outflow tract malformation, exhibiting a primary failure of the endocardial ridge differentiation into semilunar valves (Schilham et al. 1996). *Sox4* is also required for differentiation of T

and B lymphocytes (Schilham et al. 1996, 1997), pancreatic β-cells (Wilson et al. 2005) and osteoblasts (Nissen-Meyer et al. 2007). *Sox11*$^{-/-}$ mice die shortly after birth, with similar but less severe heart malformations than *Sox4*$^{-/-}$ embryos, and exhibit other defects in organogenesis (Sock et al. 2004). Analyses of compound *Sox4*$^{-/-}$ and *Sox11*$^{-/-}$ embryos demonstrate that *Sox4* and *Sox11* act partially redundantly to control key neural and mesenchymal progenitor cell fate decisions during organogenesis *in vivo* (Bhattaram et al. 2010, Thein et al. 2010, Bergsland et al. 2011, Shim et al. 2012). Compound *Sox4*$^{-/-}$*;Sox11*$^{-/-}$ kidneys have not been studied *in vivo*, due to early embryonic lethality (E8.5) of compound *Sox4*$^{-/-}$*;Sox11*$^{-/-}$ embryos. Notably, *Sox4*$^{-/-}$*; Sox11*$^{+/-}$ embryos exhibit more severe phenotypic abnormalities than *Sox4*$^{+/-}$*; Sox11*$^{-/-}$ embryos in early organogenesis, and are almost as severely affected as *Sox4*$^{-/-}$*; Sox11*$^{-/-}$ embryos. These genetic data suggest that in tissues co-expressing both *Sox4* and *Sox11*, *Sox4* may play a greater role in early developmental processes, while *Sox11* may function more strongly in later stages of development. These observations are consistent with the distinct expression pattern of *Sox4* in early nephrogenic structures and *Sox11* in more mature nephrogenic structures, collectively suggesting that *Sox4* and *Sox11* may function nonredundantly to control early and late aspects of nephrogenesis, respectively, *in vivo*.

2.4.2 Sox4 Mutant Mice Develop Postnatal Renal Disease

To begin this investigation of *SoxC* function during nephrogenesis, *Sox4* function was ablated in nephron progenitors and their cellular descendents (*Sox4*$^{nephron-}$ mice) using a well-characterized *Six2Cre (Six2TGC)* transgenic mouse strain (Kobayashi et al. 2008,

Ho et al. 2011). Analysis of *Sox4*$^{nephron-}$ embryonic kidneys did not reveal any overt abnormalities; *Sox4*$^{nephron-}$ mutant newborns appeared normal, and were born at the expected Mendelian ratios.

However, *Sox4*$^{nephron-}$ glomeruli appeared hypocellular at P7 (Figure 2.2A vs. B; Figure 2.3A vs. B), and nephron number was significantly (48%) reduced in *Sox4* $^{nephron-}$ kidneys compared to wild-type controls by acid maceration (Figure 2.2C; $P < 0.006$; n=4). Within 2 weeks of birth, *Sox4*$^{nephron-}$ mice appeared smaller than wild-type littermates, and reductions in weight became significant within 5 weeks (11.7±0.52%, $P < 0.05$, wild-type n=5; *Sox4* mutant N=9).

By P16, *Sox4*$^{nephron-}$ mutant kidneys had undergone a remarkable alteration, exhibiting a poorly organized renal medulla and cortex, and numerous dilated tubules containing periodic acid–Schiff (PAS)+ protein casts (Figure 2.3B,D, vs. A,C). Abundant PAS+ protein reabsorption granules were observed in the cytoplasm of proximal tubules of *Sox4*$^{nephron-}$ mutant kidneys (Figure 2.3D,D' vs. C,C'), evidencing substantial proximal tubular reabsorption of abnormally high levels of protein and lipoproteins in the urine, a hallmark of nephrotic range proteinuria and glomerular disease (Laurinavicius et al. 1999, Schwimmer et al. 2003). Renal corpuscles in *Sox4*$^{nephron-}$ mutants were markedly hypocellular compared to wild-type littermates and had poorly-defined glomerular tufts (Figure 2.3B'vs. A'). Characteristically thin PAS+ basement membrane staining of the glomerular capillary loops and tubular epithelium in wild-type animals is lost in *Sox4* mutants (Figure 2.3D'vs.C'), indicating damage to glomerular capillary loops,

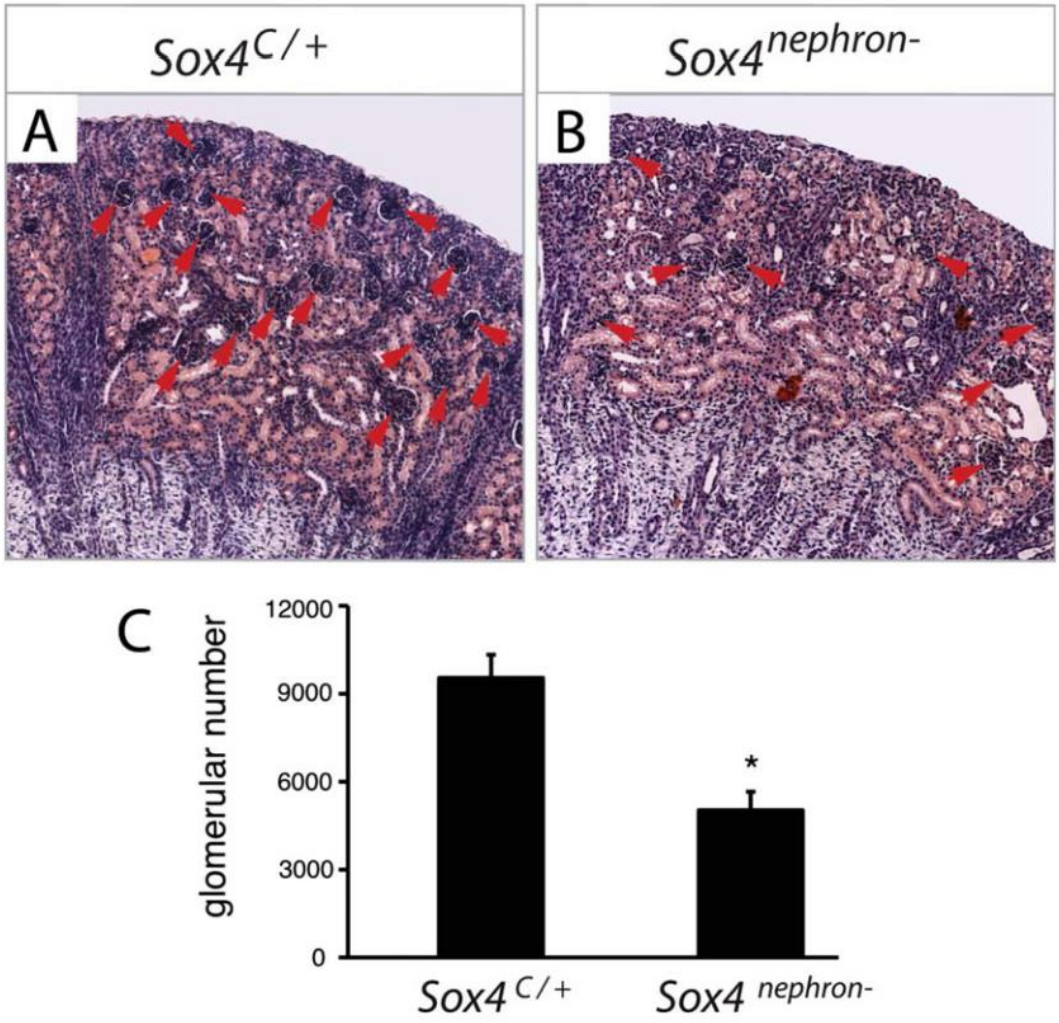

Figure 2.2. Reduced nephron endowment in Sox4$^{nephron-}$ kidneys. A,B: P7 renal histology. The number of glomeruli (arrows) was greater in control (A) versus *Sox4*$^{nephron-}$ mice (B). C: Glomerular number is significantly reduced in *Sox4*$^{nephron-}$ mice (48%) compared to wild-type littermates (*P < 0.006; n=4). Data are mean ± SE.

together with increased mesangial matrix in these animals. Collectively, these findings are consistent with early-onset proteinuria and glomerular disease in *Sox4* mutant animals.

Sox4$^{nephron-}$ mutants progress to end-stage renal disease between 5–9 months of age. Histologic analysis of *Sox4*$^{nephron-}$ mutant kidneys at 5 months revealed an abundance of

grossly dilated tubules containing protein casts as compared to wild-type controls (Figure 2.4B,D vs. A,C). Glomeruli of *Sox4*$^{nephron-}$ mutants lack clear vascular tufts, and exhibit widespread sclerosis with reduced urinary space between the parietal and visceral epithelial layers of Bowman's capsule (Figure 2.4B' vs. A'). PAS staining showed a thickening of capillary basement membranes and marked mesangial matrix accumulation in *Sox4*$^{nephron-}$ mutant glomeruli, together with focal loss of proximal tubular brush border indicative of proximal tubule injury (Figure 2.4D' vs. C'). Renal pathology was also observed by electron microscopy in these same *Sox4*$^{nephron-}$ mutant mice.

Assessment of wild-type kidneys revealed glomeruli with a normal, uniform glomerular basement membrane (GBM), and regular, interdigitating podocyte foot processes (Figure 2.5A,A'). In contrast, *Sox4*$^{nephron-}$ mutant glomeruli display extensive podocyte foot process effacement and apparent fusion characteristic of proteinuric glomerular disease (Figure 2.5B vs. A). The GBM in these mice is markedly thickened and numerous subepithelial aggregates of electron-dense deposits are present (Figure 2.5C,C'), a hallmark of membranous nephropathy (Pavelka and Roth 2005). Together, histological and ultrastructural assessment demonstrate that the structure and function of the glomerular filtration barrier is abrogated in *Sox4*$^{nephron-}$mutant mice.

The observation of reduced nephron endowment in *Sox4*$^{nephron-}$ mice demonstrates an essential role for *Sox4* in nephrogenesis *in vivo*. However, the severity of the early-onset proteinuric glomerular injury observed in postnatal *Sox4*$^{nephron-}$ mice is not easily explained by a reduction in nephron number, and suggests a requirement for *Sox4* in

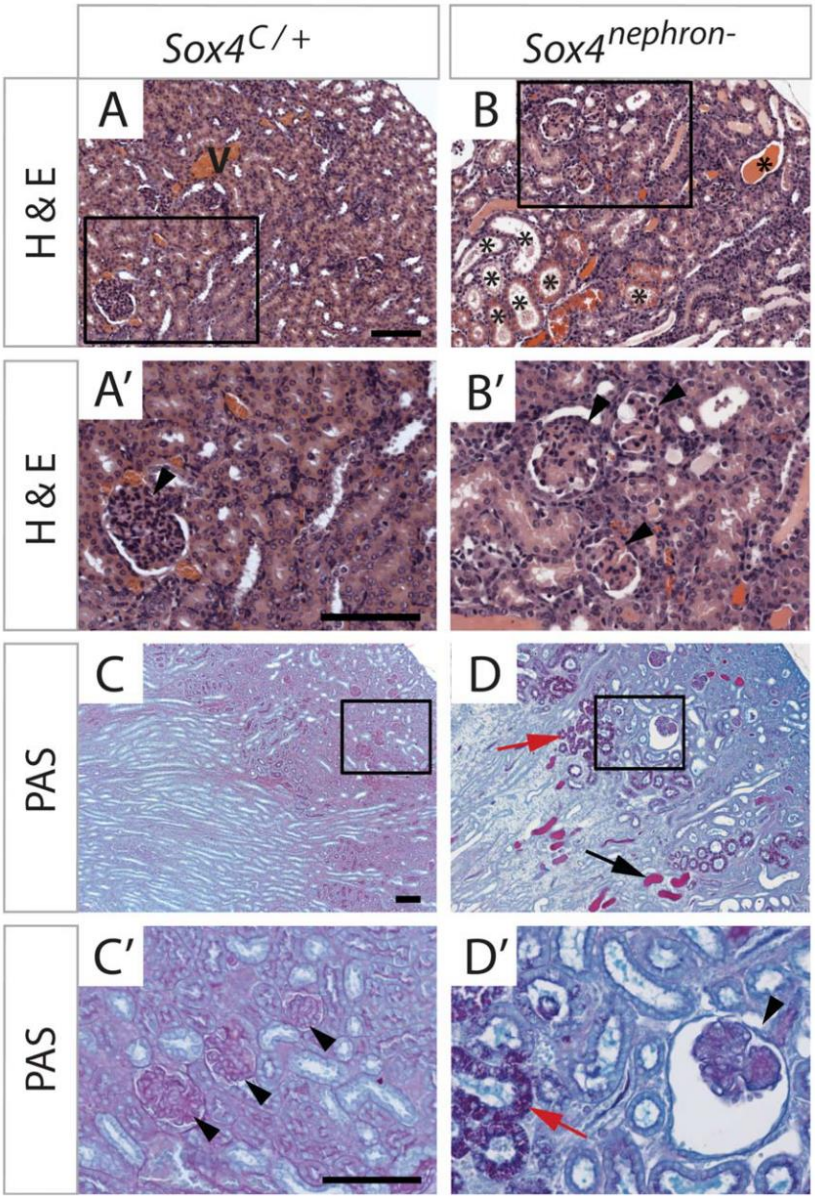

Figure 2.3. Early post-natal renal phenotype of *Sox4*$^{nephron-}$ mice. A,A',B,B': H&E stain of the renal cortex. At P16, *Sox4*$^{nephron-}$ kidneys are characterized by tubular dilatation (*), poorly defined glomerular tufts, and reduced cellularity of the renal corpuscle (arrowheads) compared to wild-type littermates (B,B' vs. A,A'). Boxes denote regions shown in high magnification. C,C', D,D': PAS stain of renal cortex and medulla. *Sox4*$^{nephron-}$ kidneys exhibit numerous dilated tubules containing PAS+ protein casts (black arrows), and proximal tubules contain abundant protein reabsorption granules (red arrows) characteristic of nephrotic range proteinuria (D vs. C). *Sox4*$^{nephron-}$ glomeruli exhibit diffuse thickening of the glomerular capillary basement membrane and increased mesangial matrix compared to wild-type littermates (D' vs. C'). v, renal vein. Scale bar = 100 μm.

podocyte development and/or adult podocyte maintenance. Given the complex and poorly-understood interplay between podocytes, endothelium, mesangium, and glomerular basement membrane, it is possible that alterations in podocyte gene expression or morphology could cause secondary responses in these cell types, which would further exacerbate glomerular injury. Indirect immunofluorescence for key glomerular markers in *Sox4*$^{\text{nephron-}}$ kidneys at E17.5 and in P7 kidneys (Figure 2.6) was performed in order to assess whether *Sox4* may regulate glomerular development and/or maintenance. At E17.5, *Sox4*$^{\text{nephron-}}$ glomeruli did not exhibit alterations in expression of early glomerular markers including glomerular basement proteins nidogen (Figure 2.6A vs. B) and β1 integrin (data not shown), glomerular mesangial markers including neural/glial antigen 2 (NG2) chondroitan sulfate (Figure 2.6E vs. F), desmin, and platelet-derived growth factor receptor (data not shown), endothelial protein platelet and endothelial cell adhesion molecule 1 (PECAM-1) (Figure 2.6I vs. J), podocyte slit diaphragm proteins nephrin (Figure 2.6M vs. N) and podocin (Figure 2.6Q vs. R), or in the podocyte nuclear marker Wt1 (Figure 2.6U vs. V). However, by P7, *Sox4*$^{\text{nephron-}}$ glomeruli exhibited a specific reduction in nephrin expression, as evaluated by immunofluorescence (Figure 2.6 O vs. P) while expression of podocin as well as other markers of the endothelium, mesangium, and basement membrane continued to be normally expressed. Nephrin is a large transmembrane protein of the immunoglobulin (Ig) superfamily, and a key structural component of the podocyte slit diaphragm

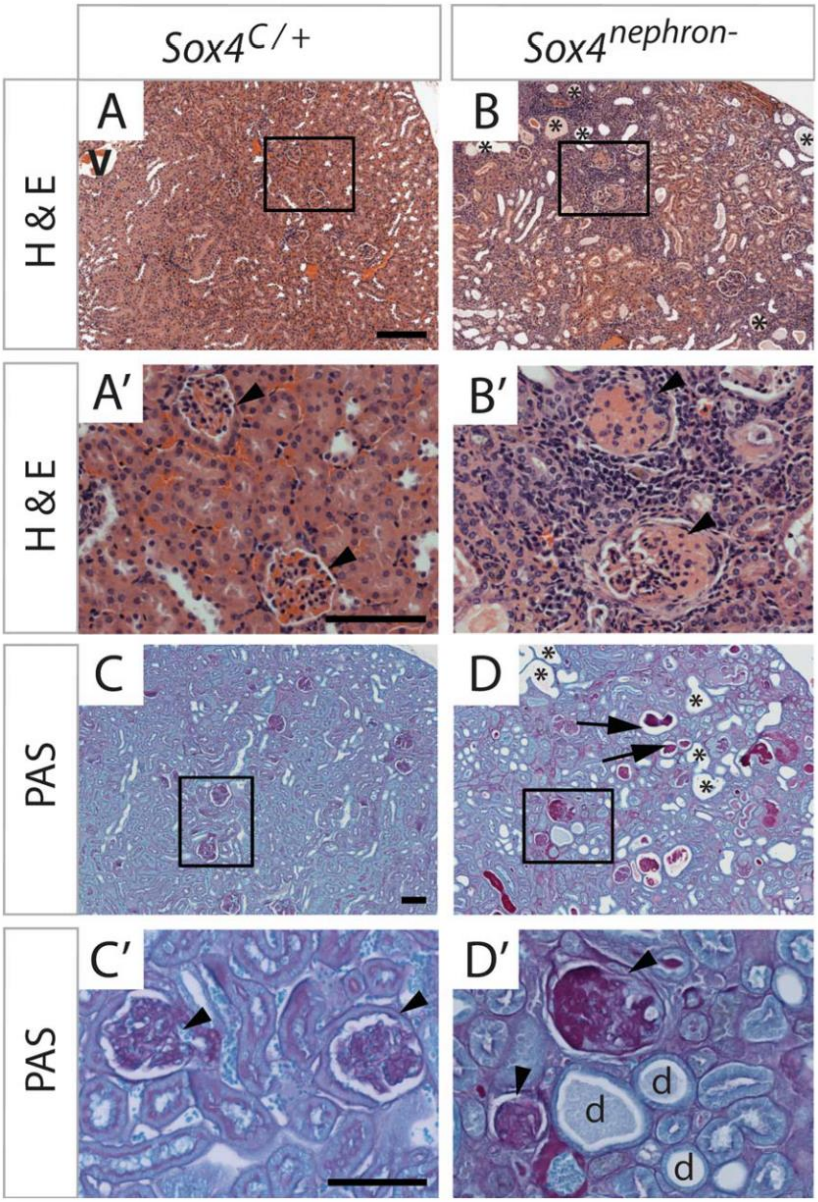

Figure 2.4. Adult (5 months) renal phenotype of *Sox4*$^{nephron-}$ mice. A–D: H&E and PAS staining of the renal cortex demonstrate the presence of numerous, large tubular dilatations (*) and protein casts (arrows) in *Sox4*$^{nephron-}$ mice compared to wild-type littermates (B,D vs. A,C). A'–D': Higher magnification of boxed areas. *Sox4*$^{nephron-}$ glomeruli (arrowheads) exhibit hypocellularity and capillary loss (B' vs. A'), as well as reduction of the urinary space between the parietal and visceral epithelia in Bowman's capsule (B',D'; vs. A',C'). *Sox4*$^{nephron-}$ glomeruli exhibit markedly thicker basement membranes and abundant PAS+ mesangial matrix compared to wild-type littermates, as well as distal tubule dilatation and loss of proximal tubular brush border (D' vs. C'). v, renal vein. Scale bar = 100 μm.

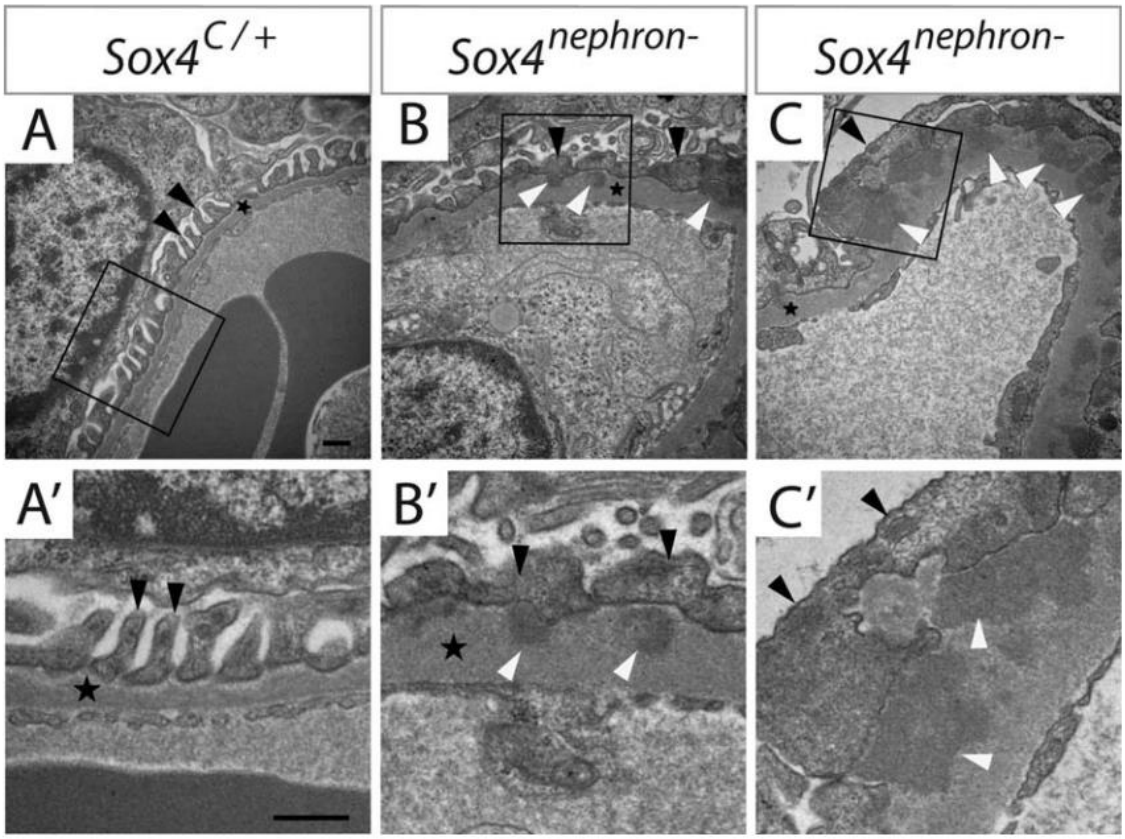

Figure 2.5. Ultrastructure of glomerular damage in adult (5 months) *Sox4*$^{nephron-}$ kidneys. A,A',B,B': *Sox4*$^{nephron-}$ glomeruli exhibit extensive podocyte injury characterized by foot process effacement (black arrow-heads) and morphologic irregularity. The glomerular basement membrane (GBM) is markedly thickened (star) in Sox4$^{nephron-}$ mice (B,B' vs. A,A'). C,C': Numerous large aggregates of subepithelial electron-dense material are present in the GBM (white arrowheads) of *Sox4*$^{nephron-}$ glomeruli. Boxes denote regions shown in high magnification in bottom panels. Scale bar = 500nm.

(Tryggvason et al. 2006). The regular spacing between adjacent podocyte foot processes is maintained through adhesive interactions between nephrin and other Ig superfamily proteins expressed on opposing foot processes. Loss or abnormal function of nephrin leads to proteinuria due to loss of the slit diaphragm and foot process effacement (Jones et al. 2009, New et al. 2013). Results from this study demonstrate reduced nephrin expression in P7 *Sox4*$^{nephron-}$ glomeruli, suggesting that loss of nephrin expression may partly underlie the observed glomerular phenotype observed in P16 *Sox4*$^{nephron-}$ mice.

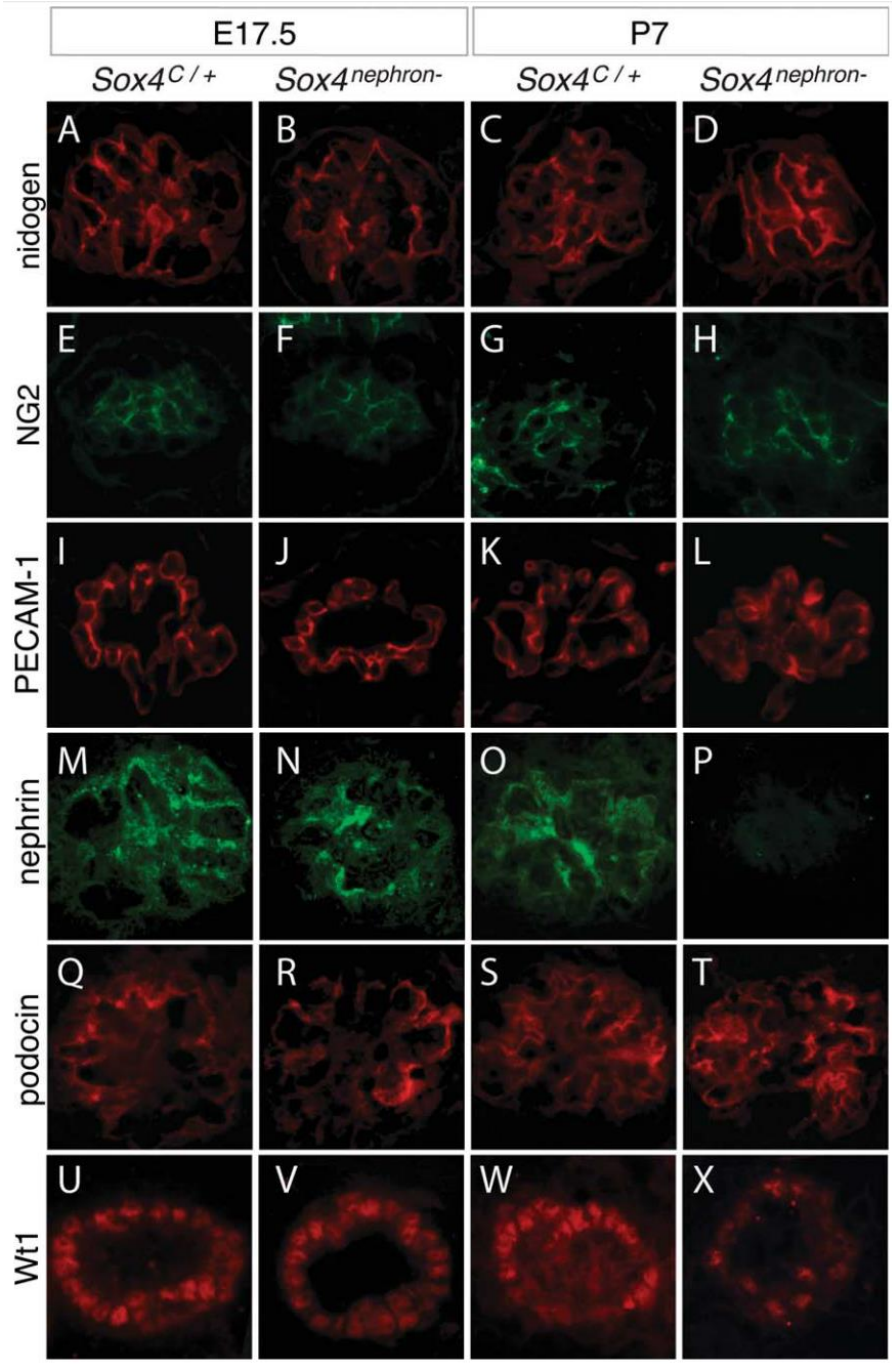

Figure 2.6. Expression of endothelial, mesangial, podocyte, and GBM components in *Sox4*$^{nephron-}$ versus control littermates at E17.5 and P7. Expression of (A–D) basement membrane protein nidogen, (E–H) mesangial marker NG2, and (I–L) endothelial protein PECAM-1 was not altered in *Sox4*$^{nephron-}$ kidneys. M–P: Expression of the slit diaphragm protein nephrin is reduced in P7 *Sox4*$^{nephron-}$ glomeruli, while (Q–T) podocin expression is unchanged compared to wild-type controls. U–X: Expression of the podocyte nuclear marker WT1 is reduced in P7 *Sox4*$^{nephron-}$ kidneys. Representative images are shown from at least 15 glomeruli examined per kidney (n=3).

The number of WT1+ podocyte nuclei is also reduced in *Sox4*$^{nephron-}$ kidneys at P7 as evaluated by immunofluorescence (Figure 2.6X vs. W). Nephrin is a transcriptional WT1 target (Wagner et al. 2004). Alternatively, loss of nephrin expression in *Sox4*$^{nephron-}$ kidneys may be secondary to reduced WT1 expression in *Sox4*$^{nephron-}$ podocytes, suggesting a role for *Sox4* in regulation of WT1 in podocytes. Importantly, reduced numbers of WT1+ podocytes in *Sox4*$^{nephron-}$ renal corpuscles may reflect reduced podocyte numbers in these mice, suggesting that aberrant nephrin expression and glomerular injury may be partly attributable to podocyte loss in *Sox4*$^{nephron-}$ kidneys, supporting a role for Sox4 in podocyte development *in vivo*.

In summary, this study has defined the relative abundance and subcellular distribution of the three *SoxC* genes during renal development. Postnatal analysis of *Sox4* mutant mice demonstrates an essential requirement for at least one member of the *SoxC* sub-family in nephrogenesis *in vivo*, and suggests potential roles for *Sox4* in podocyte development and/or adult podocyte maintenance. Future studies will assess whether *Sox4* transcriptionally regulates nephrin expression *in vitro*. Conditional gene deletion studies will be aimed at ablating *Sox4* in podocytes to determine the specific role of *Sox4* in the podocyte lineage, and to evaluate the independent role of *Sox11* as well as the combined role of *Sox4/Sox11* signalling during renal development *in vivo*.

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3. Estimation of total glomerular number using an integrated disector method in embryonic and postnatal kidneys

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3.1 Abstract

Congenital Anomalies of the Kidney and Urinary Tract (CAKUT) are a polymorphic group of clinical disorders comprising the major cause of renal failure in children. Included within CAKUT is a wide spectrum of developmental malformations ranging from renal agenesis, renal hypoplasia and renal dysplasia (maldifferentiation of renal tissue), each characterized by varying deficits in nephron number. First presented in the Brenner Hypothesis, low congenital nephron endowment is becoming recognized as an antecedent cause of adult-onset hypertension, a leading cause of coronary heart disease, stroke, and renal failure in North America. Genetic mouse models of impaired nephrogenesis and nephron endowment provide a critical framework for understanding the origins of human kidney disease. Current methods to quantitate nephron number include (i) acid maceration (ii) estimation of nephron number from a small number of tissue sections (iii) imaging modalities such as MRI and (iv) the gold standard physical disector/fractionator method. Despite its accuracy, the physical disector/fractionator method is rarely employed because it is labour-intensive, time-consuming and costly to perform. Consequently, less rigorous methods of nephron estimation are routinely employed by many laboratories. In the current study an updated, digitized version of the physical disector/fractionator method (using free open source Fiji software), termed the integrated disector method is presented. This updated version of the gold standard

modality accurately, rapidly and cost-effectively quantitates nephron number in embryonic and post-natal mouse kidneys, and can be easily adapted for stereological measurements in other organ systems.

3.2 Introduction

Defects in nephrogenesis and ureteric branching result in CAKUT. With an incidence of 1:400 (Nakanishi and Yoshikawa 2003), CAKUT constitute one of the most frequent birth defects in humans, and the major cause of childhood renal failure (Pope et al. 1999, Seikaly et al. 2003). These pleiotropic malformations comprise a multitude of renal phenotypes including renal agenesis, hypoplasia and renal dysplasia, associated with obstructive or refluxive anomalies of the ureter (Weber 2012). CAKUT are predicted to have different genetic origins (Nakanishi and Yoshikawa 2003), yet the molecular pathogenesis of CAKUT is not well understood. Nephron deficiency is a hallmark feature of CAKUT. In fact, nephron number varies greatly even within the normal distribution, ranging from 7,000 to 20,000 in mice (Cebrian et al. 2004, Murawski et al. 2010, Zhong et al. 2012), and from 200,000 to 2 million in humans (Hughson et al. 2003, Hoppe et al. 2007, Bertram et al. 2011). Low nephron endowment within this normal range - though asymptomatic early in life, is associated with adult-onset hypertension (Keller et al. 2003, Reyes and Mañalich 2005, Walker et al. 2012), a disease affecting 73 million North Americans and a leading cause of coronary heart disease, stroke, and renal failure in North America (Wilkins et al. 2010, Tedla et al. 2011). Despite the fundamental importance of nephron endowment to human health and disease, only a minority of molecular mechanisms underlying nephron morphogenesis have been identified. To this end, mouse models of impaired

nephrogenesis and nephron endowment provide an important framework for understanding the molecular and developmental origins of human kidney disease.

Four main methods are currently employed to quantitate glomerular number (N_{glom}) in the kidney (Bertram 2013). The most accessible methods for estimating glomerular number by virtue of their ease of performance, relative low cost and rapidity, are (i) acid maceration and (ii) quantitation of glomeruli in a small number of histological sections. In the acid maceration method, the kidney is decapsulated, cut into small pieces and incubated in a weakly acidic solution. Tissue fragments are gently disrupted by manual aspiration which dissociates glomeruli in the suspension, and N_{glom} is counted within a known volume in a chamber similar to a hemocytometer (MacKay et al. 1987, Godley et al. 1996). Acid maceration is a rapid, simple and inexpensive means of obtaining N_{glom}; however, this method is subject to some of the same sources of counting errors and consequent low precision associated with hemocytometer use, including non-uniform suspensions and non-adherence to convention for counting glomeruli in contact with boundary lines or each other. A tacit assumption in the acid maceration method is that glomerular structural integrity is equivalent between sample groups. While not previously reported in the literature, it is feasible that disease states or genetic mutations that deleteriously affect the structural integrity of the glomerulus would render glomeruli more susceptible to acid digestion. Under such conditions, spurious differences in glomerular number could be obtained due to degradation of glomeruli in suspension, rather than true differences in glomerular number between groups.

A second commonly employed method involves evaluation of stained histological

sections of the kidney. A small number of kidney tissue sections are chosen and glomerular number is counted and reported per area of tissue, or cortex. Although straightforward to perform, it is a biased sampling method that does not provide an accurate depiction of glomerular number for the entire kidney. Using this method, a glomerulus that is longer perpendicular to the plane of sectioning has a higher probability of being sampled in any given section than would the same glomerulus orientated parallel to the plane of sectioning. The number of glomeruli counted using this method not only depends on glomerular number and density, but also on their size and shape, which are not equally distributed throughout the kidney (Samuel et al. 2005, Kanzaki et al. 2013).

The use of micro-magnetic resonance imaging (MRI), also known as high-field MRI, is a third modality that can provide quantitative measurements *in vivo* and therefore holds great potential for future human applications. MRI provides both rapid and accurate evaluation of total glomerular number in healthy kidneys (Beeman et al. 2011, Heilmann et al. 2012). However, access to micro-MRI systems is costly, rendering it less accessible to many laboratories. In addition, the accuracy of MRI is significantly reduced in kidneys with focal and segmental glomerulosclerosis (Bennett et al. 2008), due to the low retention of the MRI contrasting agent, cationic ferritin, in the basement membranes of diseased glomeruli. Thus, disease states or genetic mutations that affect the integrity of the glomerular basement membrane may alter the retention of the contrasting agents in affected glomeruli, and can spuriously skew results.

The gold standard method for estimating N_{glom} is the unbiased physical

disector/fractionator method. This stereological method involves exhaustively sectioning the kidney, and using a physical disector (a stereological probe) to count glomeruli in pairs of tissue sections. The application of stereology in biological systems and in the context of the kidney has been well-described (Howard and Reed 1998, Nyengaard 1999). Briefly, this approach uses a pair of projection microscopes, a pencil and paper to manually circle and count glomeruli (Cullen-McEwen et al. 2012b). The physical disector method is extremely accurate because it is unbiased, but is very labour-intensive to perform, requiring a full day for an experienced technician to complete quantitation of a single rat kidney (Bertram 2013). Commercially-available software packages offer powerful stereological alternatives to using projection microscopes for the physical disector/fractionator method (Cullen-McEwen et al. 2012b), but are cost-prohibitive to most laboratories. It is also interesting to note that although the mathematics and theory of unbiased stereology have come a long way in the past several decades, the actual technical methods for sampling the number of glomeruli presented by Cullen-McEwan et al. (2012b) somewhat resemble those used by Miller and Carleton in 1894 with the use of a camera lucida in place of projection microscopes (Miller 1894)

Members of the *Sry-Related HMG Box (Sox)* gene family have been shown to play master regulatory roles in a multitude of developmental processes including dorso-ventral patterning (Chen et al. 2012), stemness (Avilion et al. 2003, Masui et al. 2007, Wang et al. 2012), male differentiation (Koopman et al. 1991, Wagner et al. 1994), neurogenesis (Bhattaram et al. 2010, Bergsland et al. 2011, Mu et al. 2012), cardiac outflow tract, B-lymphocyte (Schilham et al. 1996) and spinal cord development

(Shim et al. 2012), and skeletogenesis (Akiyama and Lefebvre 2011, Lefebvre and Bhattaram 2016). Recently *Sox4* has been identified as a critical regulator of normal renal development *in vivo* (Huang et al. 2013). Using an established *Six2-Cre* transgenic mouse line (Kobayashi et al. 2005, 2008), *Sox4* function was ablated in nephron progenitors and their cellular descendants ($Sox4^{nephron-}$ mice). Using the acid maceration method, it was demonstrated that $Sox4^{nephron-}$ kidneys exhibit 48% reduction in nephron number compared to wild-type animals. In light of the low precision associated with this method and the inherent limitations associated with each of the existing methods of glomerular quantitation, I sought to develop an accurate, rapid and cost-effective alternative means of quantitating nephron number that could be of use to the nephrology community.

ImageJ is an open source image processing program through the US National Institutes of Health which has been freely available for more than 15 years (Schneider et al. 2012). Fiji is a free biological imaging package within ImageJ that comes pre-loaded with the TrakEM2 plugin for morphological data mining and three-dimensional modeling, and includes a stereological disector tool (Cardona et al. 2012, Schindelin et al. 2012). By integrating the stereologic principles of the physical disector/fractionator method with automated light microscopy and ImageJ/Fiji software, I have developed an accurate, rapid, cost-effective method for quantitation of total nephron number in embryonic day (E)17.5 murine kidneys (including S-shaped bodies and maturing glomeruli), and estimation of nephron number in postnatal day (P)7 murine kidneys. Thus, I present the integrated disector method as a highly accessible updated version of the physical disector/fractionator method that can be easily adopted for in house application with

minimal set up costs.

3.3 Materials and Methods

3.3.1 Mouse Strains

The *Sox4* null (*Sox4*$^{-}$) allele (Schilham et al. 1996), conditional *Sox4* (*Sox4*^{fx}) allele (Penzo-Méndez et al. 2007) and *Six2-Cre BAC* transgenic line (Kobayashi et al. 2008) have been previously characterized. All animal experiments were carried out in accordance with the policies of the Animal Care Committee at the University of Prince Edward Island and according to the Canadian Council on Animal Care guidelines.

3.3.2 Histochemical Staining

Kidneys were isolated from E17.5 (n=4 *Six2Cre;Sox4*$^{fx/-}$ *(Sox4*$^{nephron-}$*); n=4 wild-type)* and P7 (n=4 *Sox4*$^{nephron-}$*; n=3 wild-type)* mice. Kidneys were fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO), processed to paraffin blocks and exhaustively sectioned in the sagittal plane at 5µm. Ribbons of serial E17.5 sections were mounted on glass slides, such that every section of the kidney was captured on a set of slides. Sections were stained with biotinylated peanut agglutinin (PNA) as described previously (Cullen-McEwen et al. 2012a) with the following modifications: reduced incubation time of the biotinylated PNA to 10 minutes and without hematoxylin counterstaining. Paired sections from P7 kidneys were selected using a systematic uniform random sampling approach as described (Cullen-McEwen et al. 2012b). Post-natal sections were stained with periodic acid-Schiff (PAS).

3.3.3 Fiji Processing of Kidney Section Images

The established workflow for embryonic kidneys was as follows (Figure 3.1): Digital

images of each stained section were captured by automated light microscopy using an Olympus BX61 Virtual Slide Microscope with motorized stage. Images were imported, converted to 8-bit grey and resized using Fiji software (Schindelin et al. 2012). All of the images for a single embryonic kidney were then imported into a single stack and registered using the TrakEM2 plugin (Cardona et al. 2012). The disector tool within the TrakEM2 environment was then used to count every glomerulus within the kidney. Individual glomeruli were tagged with a unique ID which was maintained between sections, and reports were generated.

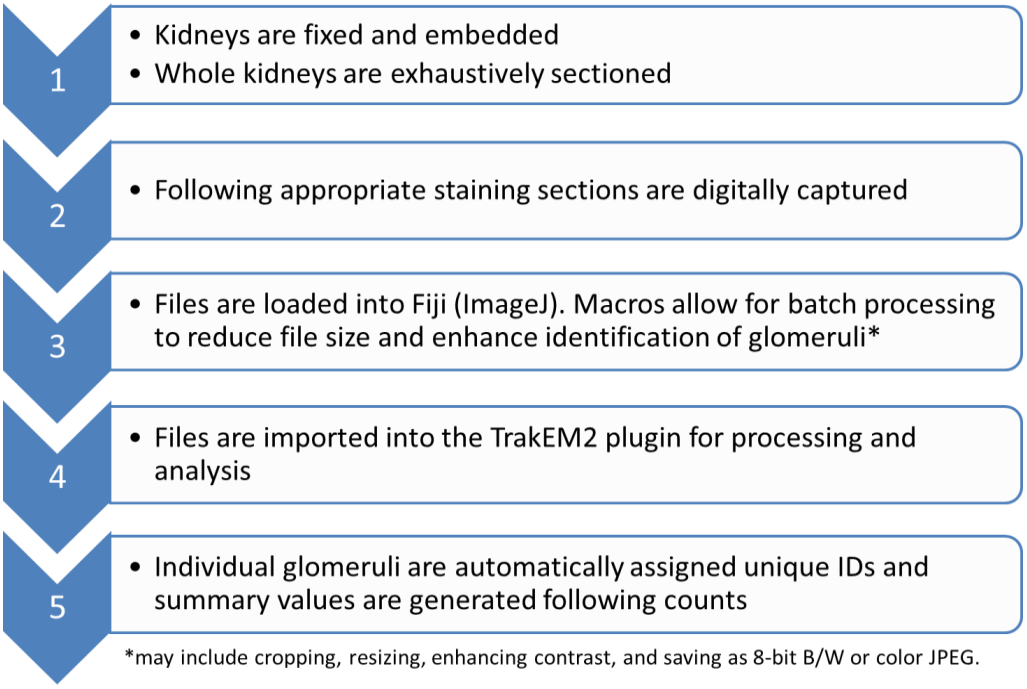

Figure 3.1. Processing of kidney section images. Schematic of workflow for quantitation of glomerular number using the integrated disector/fractionator method.

A similar approach was used to estimate glomerular number in postnatal kidneys. A pilot study was conducted to determine mean maximal glomerular size and the total number of sections through a P7 kidney, and the optimal section sampling fraction was

determined to estimate total glomerular number (please see Cullen-McEwan *et al*., 2012 for detailed protocol). Paired sections were imported, converted to 8-bit colour and manually aligned. The disector tool was then used to identify glomeruli that were either present on both paired sections or on only one of the two. Those present on only one of the two sections were counted as disector particles and used to estimate the total number of glomeruli (for more detailed technical instructions please refer to APPENDIX D).

3.3.4 Statistical Methods

Two sample t-tests were performed using Minitab 16 to compare changes in nephron endowment between *Sox4$^{nephron-}$ and wild-type mice*. Data are presented as mean ± standard deviation.

3.4 Results and Discussion

3.4.1 Assessment of Glomerular Number in *Sox4$^{nephron-}$* Kidneys

Given the inherent limitations associated with existing methods of glomerular quantitation, I sought to develop an accurate, rapid and cost-effective alternative means of quantitating nephron number in the *Sox4$^{nephron-}$* kidneys. As the physical disector/fractionator method provides the most accurate quantitative measurements of glomerular number in both healthy and diseased kidneys, ways to reduce the time and labour involved in this process, at low cost was sought. To this end, I opted for automated microscopy combined with free open source Fiji image analysis software to develop a highly accessible workflow that has been termed the integrated disector method. This workflow allows for the application of the physical disector/fractionator method in house and is a simple adaptation of the technical implementation of the

physical disector/fractionator method described in the literature. The integrated disector method is a straightforward, rapid, and accurate means of quantitating nephron number in both embryonic and postnatal kidneys. In E17.5 mouse kidneys, PNA-stained S-shaped bodies and glomeruli are efficiently identified and counted using the TrakEM2 plugin and Fiji software. Quantitation of total glomerular number in E17.5 kidneys of *Sox4*$^{nephron-}$ (n=4) and wild-type (n=4) mice demonstrated a 36% reduction in glomerular number (p=0.001) in *Sox4*$^{nephron-}$ kidneys compared to wild-type littermates (Figure 3.2a). In P7 kidneys, glomeruli were identified using PAS staining. A disector was used to obtain accurate unbiased estimates of total glomerular number from pairs of sections using the TrakEM2 plugin and Fiji software. Estimation of total glomerular number in P7 kidneys of *Sox4*$^{nephron-}$ (n=4) and wild-type mice (n=3) demonstrated a 32% reduction in glomerular number in *Sox4*-deficient kidneys compared to wild-type (p=0.012) (Figure 3.2b). The integrated disector method obtained glomerular numbers similar to those reported in the literature for both embryonic and postnatal wild-type kidneys (Cebrian et al. 2004).

Using acid maceration, it was previously reported that there was a substantially greater reduction (48%) in glomerular number in *Sox4*-deficient kidneys at P7 (Chapter 2). This difference is likely attributable to the high variability (low precision) inherent to the acid maceration technique. Variability in the acid maceration technique of glomerular quantitation is introduced by (i) pipetting errors (ii) non-uniform suspension of glomeruli and most significantly (iii) high standard deviation and its derivative coefficient of variability (CV) arising from the extrapolation of N_{glom} from a small volume (500 µL) to N_{glom} in the total volume (30 mL)(Shapiro 2003). Indeed,

quantitation of N_{glom} using acid maceration is associated with a high CV in both wild-type kidneys (12.7%) and in *Sox4* mutant kidneys (15.6%), which may largely

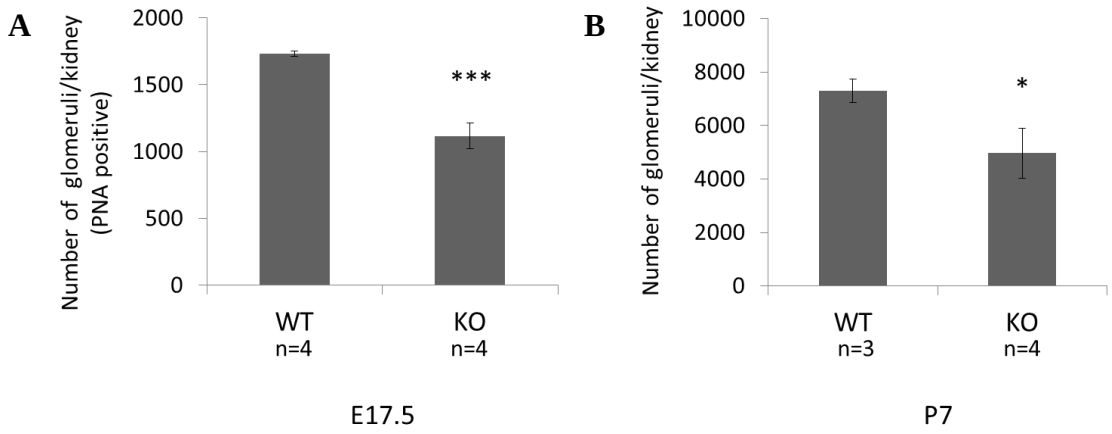

Figure 3.2. Reduced nephron endowment in *Sox4*$^{nephron-}$ (KO) kidneys. A) Total glomerular number is significantly reduced in *Sox4*$^{nephron-}$ kidneys (36%) compared to wild-type littermates (WT) at E17.5 (***p=0.001; wild-type n=4; *Sox4*$^{nephron-}$ n=4). B) Glomeruli number is significantly reduced in a *Sox4*$^{nephron-}$ kidneys (32%) compared to wild-type littermates at P7 (*p=0.012; wild-type n=3; *Sox4*$^{neprhon-}$ n=4).

explain the difference in N_{glom} obtained using acid maceration *vs*. integrated disector methodologies. When restricted by cost and resources, acid maceration continues to provide useful insight into kidney development and physiology. This approach lends itself particularly well in instances where large between-group differences exist, as in the case of *Sox4*-mutant *vs.* wild-type glomerular number, and has recently been used for validation of new MRI approaches (Beeman et al. 2011). However, it is generally accepted and understood that stereology is a more robust approach for quantification of glomeruli. Indeed, the high variability inherent to the acid maceration technique was a significant motivation for us to implement cost-effective in house stereological methods for glomerular quantitation.

3.4.2 The Integrated Disector Method is Cost-Effective and Accessible

Several open source biomedical image analysis software packages have recently become available including Endrov (Henriksson et al. 2013), Icy (Chaumont et al. 2012), BioImagXD (Kankaanpää et al. 2012) and Fiji (Schindelin et al. 2012). Each package has a specialized focus and toolset to facilitate processing of complex biological spatio-temporal image data obtained mainly by microscopy. While developing the approach presented here, multiple avenues were explored, each with their own advantages and disadvantages before we settled on the use of trakEM2 and Fiji/ImageJ, including the use of various software packages such as IMOD (Kremer et al. 1996), various machine-learning algorithms and approaches (Sommer and Gerlich 2013) and the use of other Image J plugins, including Trainable Weka Segmentation. Certainly, multiple strategies can be developed to perform glomerular quantitation with high accuracy, speed and economy. However, to the best of my knowledge, this is the first study to report a means of combining the high accuracy of the physical disector/fractionator gold standard method with the power and economy of open source image analysis software.

Although many groups are studying the effects of genes and various environmental factors on the development and function of the kidney, there is currently no broadly accessible method for accurately determining if there is an effect on nephron endowment. In the present study, PNA and PAS staining of histological sections were optimized to identify glomeruli in embryonic and postnatal kidneys. The implementation of the physical disector/fractionator method has been updated by

combining semi-automated microscopy image capture with Fiji/TrakEM2 to streamline data collection and image analysis. With minimal setup costs, these tools can permit accurate, efficient and rapid stereological measurement of glomerular number in many laboratories, and can be applied from early embryonic development through to the adult mouse. Implementing the integrated disector requires laboratories to follow the same steps associated with the physical disector/fractionator method from tissue embedding to exhaustive sectioning and staining of tissue (Cullen-McEwen et al. 2012b). The power of this approach is in its ease of set up and accessibility for counting N_{glom} using a freely available disector tool which allows for unbiased estimation of N_{glom}. Total time for image acquisition and analysis for an experienced user and full automation would average 2-3 hours per kidney. Smaller laboratories that may not have access to motorized microscopes can easily outsource the image capture component to collaborators while performing the Fiji/TrakEM2 quantitation in-house. Alternatively, researchers can manually capture overlapping partial images of serial sections at the appropriate magnification, and sections can be stitched using the Grid/Collection stitching plugin built-in to Fiji. Stitched sections can then be loaded into the TrakEM2 environment and analysed as described. Other refinements and adaptations can be performed to best suit the needs of individual research teams, including the acquisition of volumetric data, and can be broadly applied towards stereological measurements in other organ systems.

In summary, the physical disector/fractionator method of stereological glomerular quantitation has been adapted using semi-automated microscopy together with open

source Fiji software and the TrakEM2 plugin. The integrated disector method is presented in the hopes of offering the general nephrology community an accurate, rapid, cost-effective and therefore highly-accessible means of glomerular quantification in embryonic and postnatal kidneys.

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4. *Sox4* in kidney development: an exploration of possible mechanisms through preliminary data

4.1 Abstract

The transcription factor Sry-Related HMG Box-4 (*Sox4*) has been shown to be essential for normal renal development *in vivo.* In Chapters 2 and 3, it was shown that conditional ablation of *Sox4* in nephron progenitors and their cellular descendants ($Sox4^{neprhon-}$ mice) results in a significant reduction in nephron endowment. These mice also develop early-onset proteinaceous glomerular injury which progresses to end-stage renal failure within 5-9 months. In this study the reduced nephron endowment in $Sox4^{neprhon-}$ mice is shown to result from the formation of fewer pretubular aggregates, likely as a result of decreased cell cycling of nephron progenitor cells. *Sox4* and *Sox11* are also dispensable for normal podocyte differentiation and function. Conditional deletion of both of these genes individually as well as in combination in podocytes resulted in no apparent glomerular phenotype after 6 months and does not recapitulate the phenotype observed in $Sox4^{neprhon-}$ kidneys. Analysis of $Sox4^{neprhon-}$ kidneys suggests that the genesis of glomerular injury may be activated parietal epithelial cells (aPEC). Collectively, these results demonstrate that *Sox4* plays multiple crucial roles in normal kidney development and function *in vivo.*

4.2 Introduction

Kidney development results from a complex series of reciprocal signaling events. As the ureteric bud (UB) invades the metanephric mesenchyme (MM) and reciprocal signaling ensues, the cells of the MM that are closest to the UB tips condense and form a distinct subdomain of the MM, often referred to as cap mesenchyme. These Six2+ cells

represent a nephron-committed, self-renewing progenitor population that is responsible for generating all the segments of the functioning nephron (Figure 4.1). Briefly, the committed cells begin to cluster into pretubular aggregates (PTA) which further differentiate and epithelialize to form the renal vesicle (RV). The epithelial RV then forms a lumen and unwinds to sequentially form comma-shaped and S-shaped bodies. The layers of Bowman's capsule (BC) (parietal layer, Bowman's space, visceral layer, filtration barrier) are generated from the proximal RV in conjunction with the attraction of angioblasts from the surrounding interstitium to the proximal RV to form the glomerular capillaries. The distal RV fuses with the UB tip and further proliferates and differentiates to form the various tubular segments of the functioning nephron (Little and McMahon 2012, McMahon 2016).

The podocyte is a highly specialized epithelial cell that covers the glomerular capillaries, representing the visceral layer of Bowman's capsule, and has been the focus of significant research in normal and pathological conditions for several decades (Shankland et al. 2014). A unique characteristic of the podocyte is the network of interdigitated foot processes with their slit diaphragms, that along with the glomerular basement membrane (GBM) and underlying fenestrated endothelium of the glomerular capillaries, make up the glomerular filtration barrier (Barisoni and Mundel 2003). The loss of integrity of the filtration barrier leads to proteinuria and disease (Menon et al. 2012).

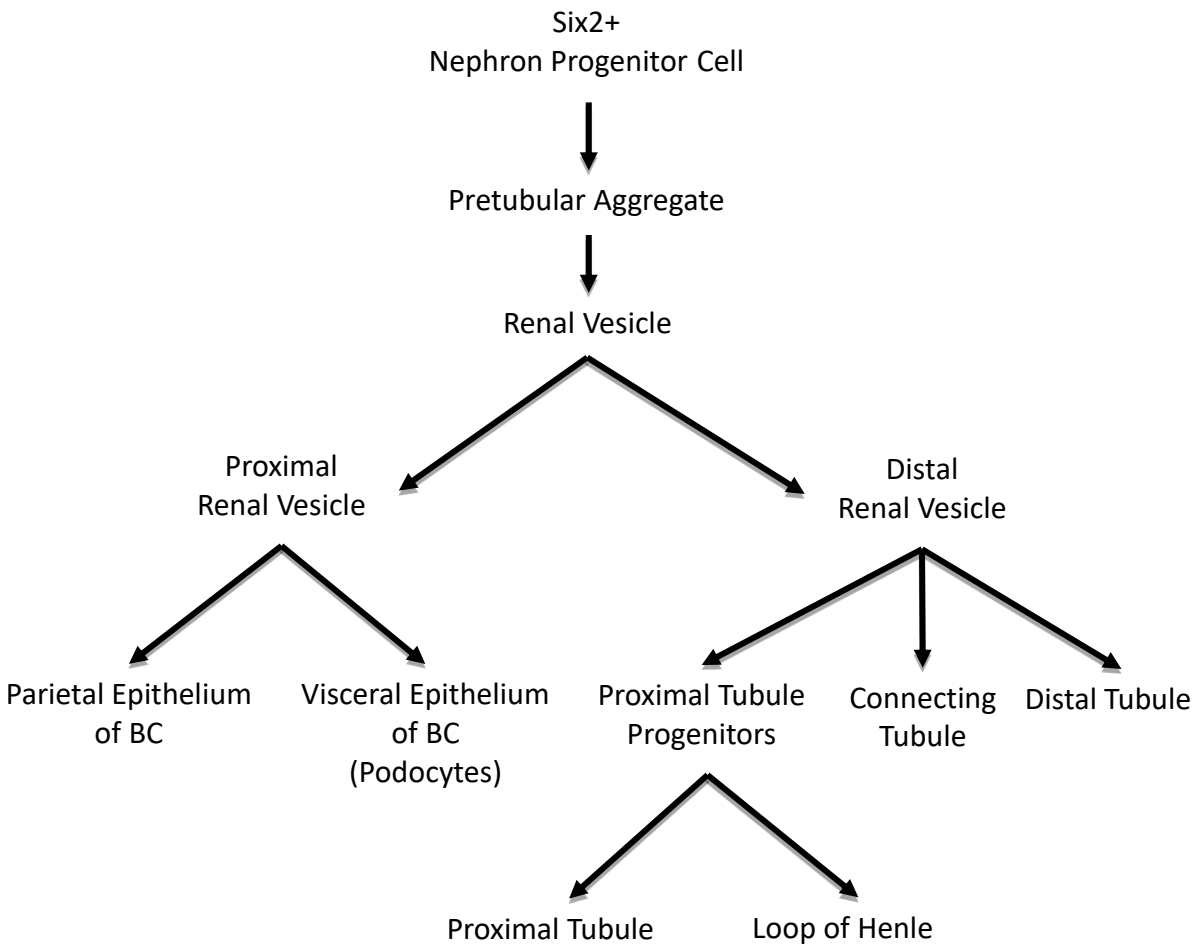

Figure 4.1. Lineage relationship of nephron progenitor cells and their derivative structures and cells types in the kidney. BC – Bowman's capsule. Adapted from Little and McMahon 2012.

The Wilms' Tumour Suppressor-1 (***Wt1***) gene, which encodes a DNA- and RNA binding nuclear transcription factor (Haber et al. 1993, Rivera and Haber 2005), is well characterized as a critical player in kidney development and function. In mice, homozygous deletion of *Wt1* results in bilateral renal agenesis (complete absence of kidney formation), characterized by a primary failure of nephron progenitor cells (NPC) to differentiate in response to inductive signals from the UB (Kreidberg et al. 1993,

Kreidberg 2002). The expression of *Wt1* is essential for nephrogenesis as well as maintenance of renal function in the adult (Guo et al. 2002). WT1 has also been identified as having a central role in podocyte differentiation, and almost half of the podocyte specific genes identified by a chromatin immunoprecipitation coupled to microarray (ChIP-chip) analysis are direct targets of WT1 (Lefebvre et al. 2015). Nevertheless, the molecular mechanisms that mediate *Wt1* function in the developing kidney remain largely undefined.

In an effort to understand the role of WT1 in the kidney, ChIP-chip analysis identified all three members of the Sry-related high mobility group (HMG) box (Sox)-C subfamily (composed of *Sox4*, *Sox11* and *Sox12*) of nuclear transcription factors as novel WT1 target genes in the embryonic mouse kidney (Hartwig et al. 2010). In the absence of *Sox4* in NPC (*Sox4*$^{nephron-}$), a significant reduction in nephron endowment is observed along with a marked thickening of the GBM and extensive podocyte foot process effacement. Further, these mice develop severe proteinaceous kidney injury within 2 weeks of birth, leading to end-stage renal disease.

In this study reduced nephron endowment in *Sox4*$^{nephron-}$ mice results from the formation of fewer pretubular aggregates, likely as a result of decreased cell cycling of nephron progenitor cells. As the severity of the phenotype observed in the kidneys of *Sox4*$^{nephron-}$ mice cannot result solely from a reduction in nephron number, the role of *Sox4* and *Sox11* in podocytes was investigated. Conditional deletion of both of these genes individually as well as in combination in podocytes were generated using the 2.5P-Cre, which expresses Cre recombinase under the control of a 2.5kb fragment of the human

NPHS2 (podocin) gene (Moeller et al. 2003). No apparent glomerular phenotype was observed after 6 months and integrity of the glomerular filtration barrier was maintained in these animals demonstrating that *Sox4* and *Sox11* are dispensable for normal podocyte differentiation and function *in vivo*. The identification of parietal epithelial cells (PEC) extending towards the capillary tuft, along with the presence of multiple layers of PEC and increased number of PEC in adult *Sox4*$^{nephron-}$ glomeruli suggests that the genesis of glomerular injury may be the result of activation of PEC. Collectively, these results further demonstrate that *Sox4* plays an essential role in establishing nephron endowment, as well as suggesting a role in regulating the activation of PEC *in vivo*.

4.3 Materials and Methods

4.3.1 Mouse Strains

The *Sox4* null (*Sox4*$^{-}$) allele (Schilham et al. 1996), conditional *Sox4* (*Sox4*^{fx}) allele (Penzo-Méndez et al. 2007), *Six2-Cre BAC* transgenic line (Kobayashi et al. 2008), 2.5P/NPHS2-Cre (Podocin-Cre) transgenic line (Moeller et al. 2003) and ROSA26 reporter strain (Soriano 1999) have been previously characterized. All animal experiments were carried out in accordance with the policies of the Animal Care Committee at the University of Prince Edward Island and according to the Canadian Council on Animal Care guidelines.

4.3.2 Estimation of Glomerular Number

Glomerular number was estimated using the integrated disector method as described in Chapter 3. Briefly, kidneys (n=5 *Six2Cre;Sox4*$^{fx/-}$ *(Sox4*$^{nephron-}$*); n=3 wild-type)* were dissected from male mice at 30 days of age. Kidneys were fixed in 10% neutral buffered

formalin (Sigma-Aldrich, St. Louis, MO), processed to paraffin blocks and exhaustively sectioned in the sagittal plane at 5µm. Paired sections were collected using a systematic uniform random sampling approach, stained with periodic acid-Schiff (PAS), and glomeruli were identified and counted.

4.3.3 Explant Experiments

E12.5 kidneys were dissected out on ice and placed on a 0.4µm pore size cell culture insert (Becton Dickinson, Mississauga, ON Canada). The insert was then placed in a 12 well polystyrene cell culture plate on top of 400µL of improved minimum essential medium with Zinc (Gibco, Grand Island, NY) supplemented with 50µg/mL transferrin (Gibco). Explants were maintained in the incubator (37°C, 5% CO_2) for 72 hours, changing the media every 24 hours. Explants were then fixed with cold methanol, blocked with 2% Bovine Serum Albumin (Sigma-Aldrich) in 0.1% Phosphate-buffered saline (PBS)-Tween (Bio-Rad, Hercules, CA) and stained with anti-WT1 (Santa Cruz sc-192) and cytokeratin (Sigma C9687-30).

4.3.4 BrdU and TUNEL Assay

For BrdU labeling, timed pregnant female mice were injected intraperitoneally with BrdU labeling reagent (Roche, Penzberg, Germany), at a dose of 30µg/g body weight. Pregnant mice were euthanized by CO_2 overdose and cervical dislocation 1 hour later. Kidneys were dissected out and placed in 30% sucrose (Sigma-Aldrich) in PBS overnight at 4°C, then flash frozen in Tissue-TEK 4583 OCT compound (Sakura Finetek, Torrance, CA) and sectioned at 5µm. Sections were fixed for 20 minutes in 15mM Glycine Ethanol Fixative with 0.1% Saponin (Sigma-Aldrich), pH 5 at -20°C.

Following 3 x 5 minute washes with 0.1% Saponin (Sigma-Aldrich) in PBS, slides were treated with DNAse (Roche) for 5 minutes at room temperature, washed 3 x 5 minutes with 0.1% Saponin in PBS and incubated with primary antibodies, rabbit α-Six2 1:400 (Proteintech, Rosemont, IL) and mouse α-BrdU 1:10 (Roche), in incubation buffer at room temperature for 1 hour. Following 3 x 5 minute washes with 0.1% Saponin in PBS, slides were incubated with secondary antibodies, Alexa594 α-rabbit 1:400 and BrdU Kit α-mouse 1:10 for 1 hour at room temperature. Slides were then washed and mounted.

For TUNEL labeling frozen sections were generated as above and stained according to manufacturer's instructions (Roche kit) following staining with rabbit α-Six2 1:400 and Alexa594 α-rabbit 1:400 (Jackson ImmunoResearch, Westgrove, PA) . For both TUNEL and BrdU, a single section down the midline of the embryonic kidney was used for quantification.

4.3.5 Histology

Kidney tissues from adult mice were fixed in 10% neutral buffered formalin and processed to paraffin blocks. Tissues were sectioned at a thickness of 5 μm and subsequently stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS).

4.3.6 Transmission Electron Microscopy

The cortical region of the kidney was trimmed, cut into 2–3 mm thick sections, and immediately immersed in 2% glutaraldehyde (SP1 Supplies, West Chester, PA) in 0.1 M phosphate buffer, pH 7.2, and refrigerated overnight at 4°C. Tissues were washed

twice for 10 minutes with 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide (SP1) in phosphate buffer for 2 hr at room temperature, then washed for 10 min in 0.1 M phosphate buffer. Tissue was then dehydrated through a graded series of ethanols, cleared in propylene oxide (SP1), and infiltrated and embedded in Epon (SP1). Semi-thin sections (0.5 mm) were cut and stained with 1% toluidine blue in 1% sodium tetraborate solution and examined with a light microscope. All samples that contained at least two glomeruli were re-cut to generate ultrathin sections (90 nm). Ultrathin sections were stained with uranyl acetate and Sato's lead stain. Sections were examined using a Hitachi H7500 transmission electron microscope operated at 80 kV and digital images were captured using an AMT XR40 side-mounted camera.

4.3.7 X-Gal Staining

E16.5 kidneys were dissected out in ice-cold PBS, fixed in 20% formaldehyde (SP1) and 2% glutaraldehyde (SP1) for 40 minutes with shaking. Kidneys were then washed 3 times for 20 minutes in PBS and incubated overnight with staining solution (Sigma) at 37°C. Embryonic kidneys were then washed 3 times for 20 minutes in PBS and fixed overnight in 4% paraformaldehyde (Sigma-Aldrich) at 4°C. Kidneys were then processed to paraffin blocks. Tissues were sectioned at a thickness of 5 μm, cleared of paraffin with xylenes and rehydrated to water through a series of ethanols. Cover slips were then mounted with Flo-Texx (Lerner Laboratories, Pittsburgh, PA) and sections imaged.

4.3.8 Statistical Methods

Comparison of *Sox4*$^{nephron-}$ and wild-type animals was performed by two sample t-tests using Minitab 16. Data are presented as mean ± standard deviation.

4.4 Results and Discussion

4.4.1 Nephron Endowment

There is a reduction in the number of nephrons, both embryonically and postnatally, when *Sox4* expression is conditionally ablated in NPC (*Sox4*$^{nephron-}$) (Chapter 2 and Chapter 3). This reported reduction could be the result of a delay in nephrogenesis rather than a direct effect on nephron endowment. In order to confirm the role *Sox4* plays in nephron endowment, an accurate estimate of nephron number was established in adult male mice (post natal day 30) by unbiased stereology using the integrated disector method (Chapter 3). A significant reduction in nephron endowment (47%) was maintained in adult *Sox4*$^{nephron-}$ mice compared to wild-type controls (Figure 4.2).

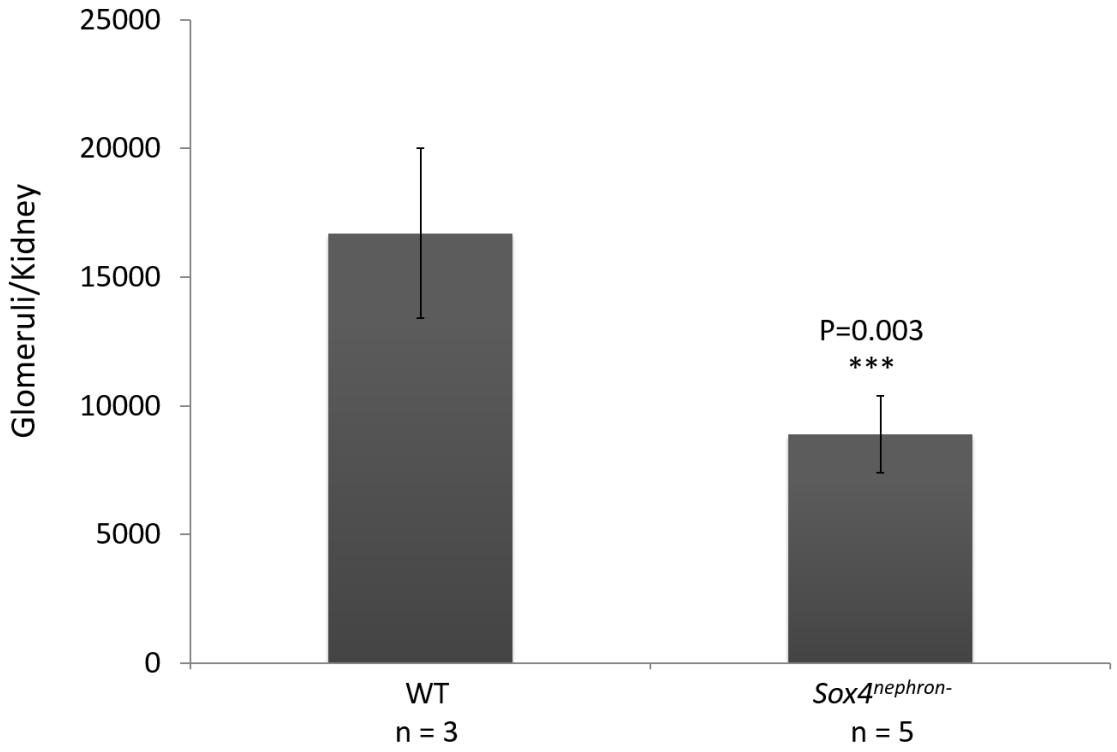

Figure 4.2. Reduced nephron endowment is maintained in adult (postnatal day 30) *Sox4*$^{nephron-}$ kidneys. Glomerular number is significantly reduced in *Sox4*$^{nephron-}$ mice (47%) compared to wild-type (WT) littermates as determined by the integrated disector method. Data are Mean ± SD.

In an effort to elucidate the genesis of the renal phenotype observed in *Sox4*$^{nephron-}$ mice, the effect of *Sox4* deletion on embryonic nephron development was evaluated by quantifying the number of nephron precursor pre-tubular aggregates (PTA) in whole organ kidney explant cultures. Embryonic day (E)12.5 kidneys were cultured for 72 hours and PTA were readily identified by WT1 immunofluorescence as large punctate structures adjacent to UB (Figure 4.3A). In the mouse, the generative fertilisation of multiple oocytes occurs within a given window of time following copulation. However, not all embryos in a given litter will be fertilized at exactly the same point in time resulting in a slight asynchrony of development among embryonic littermates. In order to account for this potential lack of synchronicity at the time of dissection, counts were normalized to UB branch points. Although the number of PTA varied greatly in both the *Sox4*$^{nephron-}$ and control explants, a highly significant reduction (47%) was observed in the a *Sox4*$^{nephron-}$ explants compared to wild-type controls (Figure 4.3B).

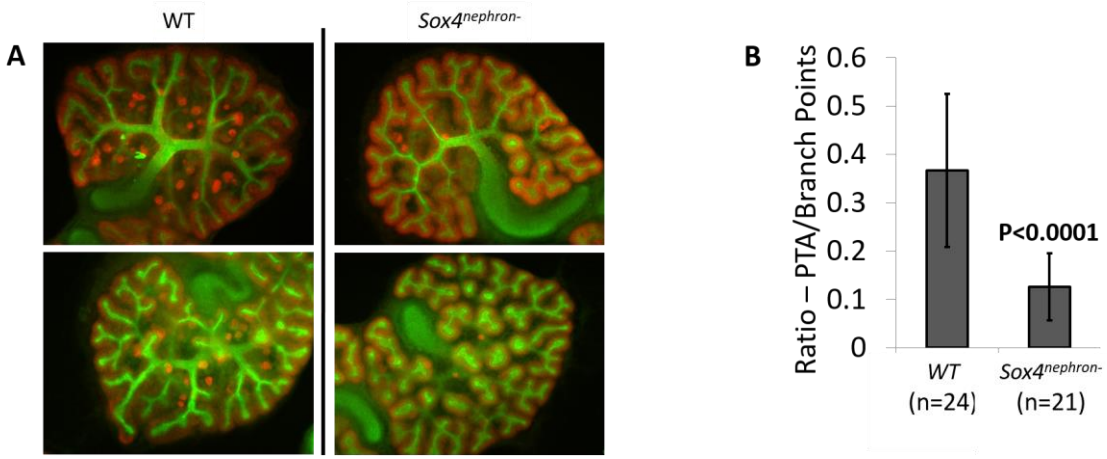

Figure 4.3. Embryonic kidney explants exhibit a 47% reduction in pre-tubular aggregate (PTA) formation in the absence of *Sox4*. A) Representative wild-type (WT) and *Sox4*$^{nephron-}$ E12.5 explants after 72 hours in culture. Red: nephron progenitor cells and pre-tubular aggregates (WT1). Green: ureteric bud (cytokeratin). B) Quantification of PTA normalized to ureteric bud branch points. Data are Mean ± SD.

Sox4 is a transcription factor that has been shown to play many roles in various organ systems, at times being identified as an inhibitor of apoptosis, while in others upregulating apoptosis depending on the cellular context (Vervoort et al. 2013). In the absence of *Sox4* in SIX2 positive NPC, the decrease in the number of nephrons both postnatally and embryonically results from the formation of fewer PTA. In the absence of *Sox4*, SIX2 positive NPC may be affected by an increase in apoptosis, a decrease in cell proliferation, a change in cell fate or some combination of the three.

In order to evaluate these possible mechanisms, protocols were established and refined to evaluate any effects on cell proliferation and apoptosis. The TUNEL assay was used to compare the number of cells undergoing apoptosis between wild-type and $Sox4^{nephron-}$ kidneys. No Six2 positive cells undergoing apoptosis were observed and preliminary data indicate that no differences in apoptosis in the nephrogenic zone were observed at E15.5 in the kidneys of conditional knockout embryos compared to controls as quantified by TUNEL assay (Figure 4.4A).

The incorporation of BrdU into newly synthesized DNA was used as a proxy marker to identify cycling cells and to compare rates of proliferation of Six2 positive cells in $Sox4^{nephron-}$ and control kidneys. Reproducibility and consistency of this assay was an ongoing issue. Several pilot studies, with small sample sizes, were undertaken using variations of the protocol. Overall, the data on all accounts indicated a trend towards a decrease in Six2 positive cycling cells in the $Sox4^{nephron-}$ kidneys compared to control (Figure 4.4B). The data suggests that the absence of *Sox4* in NPC leads to a reduction in cell cycling which, in part, results in fewer PTA and lower nephron endowment.

However, further refinement of the protocol and larger sampling are required to substantiate this finding. Further, in order to assess the possibility that NPC cell fate is altered and that proper signaling is not maintained in the absence of *Sox4*, a panel of markers covering the various cellular compartments of the kidney will need to be evaluated.

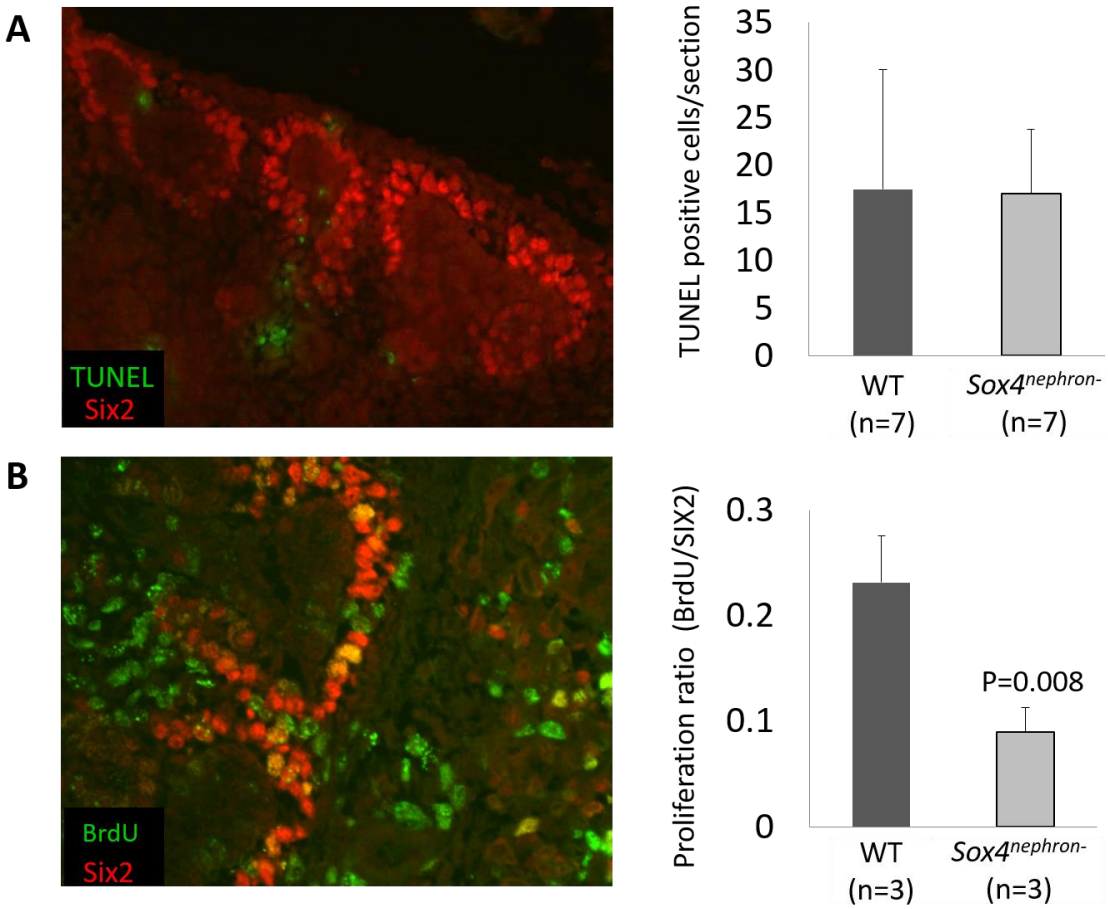

Figure 4.4. Preliminary data indicate that ablation of *Sox4* in SIX2 positive nephron progenitor cells leads to a significant reduction in cell proliferation with no effect on apoptosis in embryonic day (E)15.5 kidneys. A) Left – representative image of SIX2 and TUNEL staining in wild-type (WT) kidney. Right – Quantification of TUNEL positive cells. No significant difference is observed in TUNEL positive cells between WT and *Sox4*$^{nephron-}$ kidneys in the nephrogenic zone. B) Left - representative image of Six2 and BrdU immunostaining in WT kidney. Right – Ratio of BrdU+;Six2+ cells/ total Six2+ cells indicates a significant reduction in cell proliferation in the absence of *Sox4*. Data are Mean ± SD.

4.4.2 Conditional Knockout of *Sox4* and *Sox11* in Podocytes

It is clear that in the absence of *Sox4* in NPC, mice develop fewer nephrons, however this alone would not lead to the death of the animal as a result of kidney failure. Although very few indications exist that the kidney can self-repair, it is clear that a given number of adaptations take place in the kidney following damage. These have been observed and documented in the case of the solitary kidney following a unilateral nephrectomy (removal of a single kidney, either due to renal disease or in the case of a living kidney donor). In the case of a living donor, the number of nephrons is reduced by 50% with the removal of one kidney, as a result the size and function of the remaining kidney increases to compensate for the loss. Adaptive compensatory phenomena can be observed and include an increase in plasma flow, increased glomerular pressure, glomerular hyperfiltration accompanied by increased glomerular size and kidney mass (Gluhovschi et al. 2013). The hypertrophy and hyperfiltration observed in the remaining nephrons may lead to glomerular basement membrane lesions and further progress to proteinuric renal disease. Barring the presence of other disease conditions, such compensatory adaptations take years to develop. For this reason, the severity of the early-onset proteinuric glomerular injury observed in postnatal *Sox4*$^{\text{nephron-}}$ mice, renders a reduction in nephron number alone likely insufficient to fully explain the observed phenotype.

The absence of *Sox4* in NPC leads to the abrogation of the structure and function of the glomerular filtration barrier, which is characterized by a marked thickening of the GBM and extensive podocyte foot process effacement (reported in Chapter 2). In wild-type mice, *Sox4* expression was present in epithelialized nephrogenic structures, including

the renal vesicle and putative podocytes (Chapter 2). As podocytes are derived from NPC (Figure 4.1), an investigation into the requirement for *Sox4* in podocyte development and maintenance was warranted. It is possible that a primary defect in podocyte development or maintenance could lead to the loss of integrity observed in the filtration barrier. However, this could also occur secondary to a primary insult not intrinsic to the podocytes. In order to clearly distinguish between these two options, *Sox4* function was ablated in podocytes (*Sox4*$^{podocyte-/-}$) using the 2.5P-Cre, which expresses Cre recombinase under the control of a 2.5kb fragment of the human NPHS2 (podocin) gene (Moeller et al. 2003).

The expression of the Cre recombinase was confirmed by crossing the 2.5P-Cre mice with the ROSA26 reporter strain (Soriano 1999) and evaluating LacZ expression through β-galactosidase activity in E16.5 kidneys. In the absence of 2.5P-Cre, no β-galactosidase activity was detected throughout the kidney and the glomeruli remained unstained by X-Gal (Arrows, Figure 4.5). In the presence of 2.5P-Cre, X-gal staining is present in all mature glomeruli, confirming previous reports of the specificity and high efficiency of Cre excision (Moeller et al. 2003).

In conjunction with the generation of mice lacking *Sox4* function in podocytes, an evaluation of the role in podocytes of *Sox11*(*Sox11*$^{podocyte-/-}$) and the combined role of *Sox4/Sox11* (*Sox4*$^{podocyte-/-}$;*Sox11*$^{podocyte-/-}$) was needed by generating the associated conditional and double conditional knockout mice. Mice from all three strains were born in the expected Mendelian ratios and no distinctions could be made between wild-type and conditional knockouts in regards to size at birth or weight gain over 6 months (data

not shown). Kidneys from male littermates were evaluated by light microscopy at 6 weeks and 6 months of age. No significant changes associated with genotype were identified in either *Sox4*$^{podocyte-/-}$ or *Sox11*$^{podocyte-/-}$ kidneys when compared to littermate controls (data not shown). For the double conditional knockout strain, 5 distinct genotypes were evaluated: *Sox4*$^{podocyte-/-}$*;Sox11* $^{podocyte-/-}$, *Sox4* $^{podocyte-/-}$*;Sox11* $^{podocyte+/-}$, *Sox4* $^{podocyte+/-}$*;Sox11* $^{podocyte-/-}$, *Sox4* $^{podocyte+/-}$*;Sox11* $^{podocyte+/-}$, and wild-type controls. No significant changes associated with genotype were identified in any of the 4 genotypes of the double conditional knockout strain by hematoxylin and eosin or PAS staining when compared to littermate controls at 6 weeks (data not shown) and 6 months (Figure 4.6). All microscopic findings were reviewed by a board-certified (American College of Veterinary Pathologists) veterinary pathologist.

Evaluation of the structure of the glomerular filtration barrier and podocytes was carried out by transmission electron microscopy. At 6 months of age, no significant differences associated with genotype were observed in any of the genotypes evaluated. Podocyte structure and foot processes were conserved, slit diaphragms were present and GBM thickness was maintained in the absence of *Sox4* (Figure 4.7A), *Sox11* (data not shown) and *Sox4/Sox11* (Figure 4.7B) in podocytes. Although Cre was properly expressed, as determined by ROSA26 reporter strain, no apparent glomerular phenotype was observed after 6 months and integrity of the glomerular filtration barrier was maintained. As it has previously been shown that *Sox4* expression is significantly reduced in the adult kidney, it was hypothesized that the primary role of *Sox4* in the kidney is during embryonic development. The phenotype observed in *Sox4*$^{nephron-}$ kidneys is likely the result of congenital defects further aggravated by a significant decrease in nephron number which

ultimately leads to end-stage kidney disease (ESKD). It has been demonstrated here that the absence of *Sox4* expression in podocytes does not recapitulate the phenotype

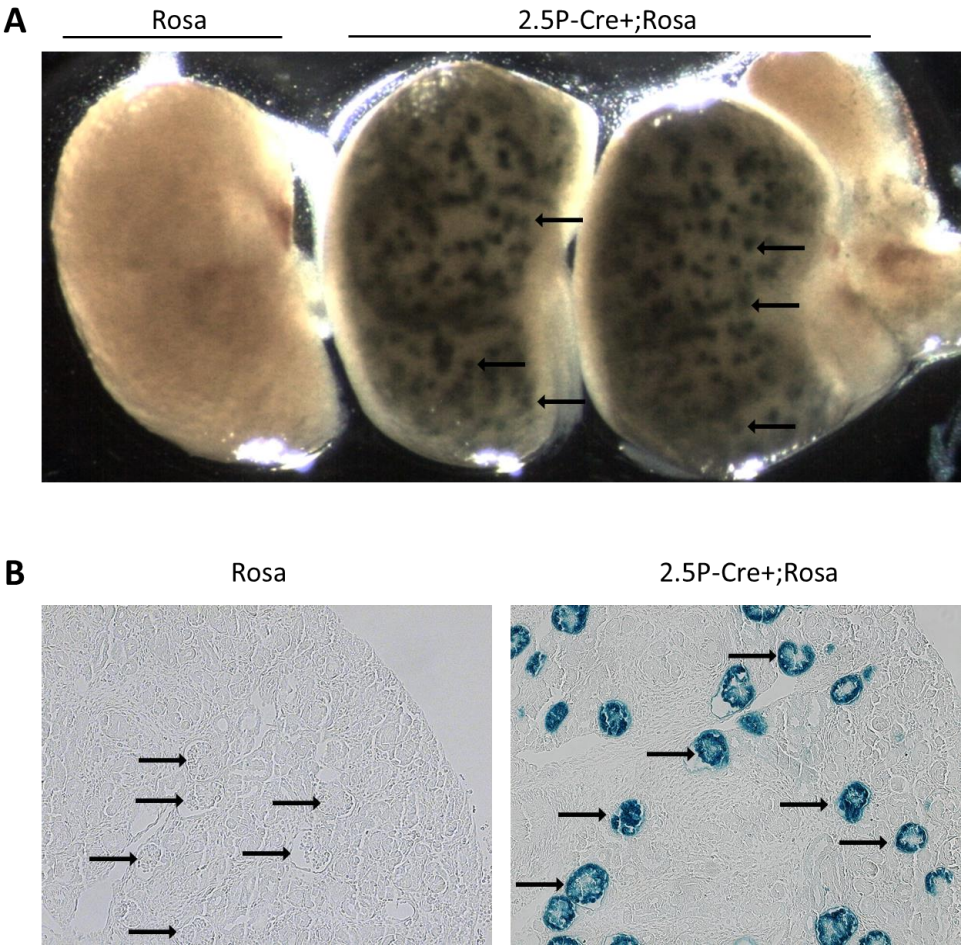

Figure 4.5. Enzymatic X-gal staining confirms expression of 2.5P-Cre in podocytes of the developing kidney. A) Whole kidneys stained with X-gal at E16.5. Left – in the absence of the 2.5P-Cre transgene, kidney remains unstained, while in the presence of the transgene (middle and right kidney) glomeruli stain blue (arrows). B) 5µm sections of kidneys in A. Left – in the absence of the 2.5P-Cre transgene, mature glomeruli (arrows) remain unstained, while in the presence of the transgene (right) glomeruli stain blue.

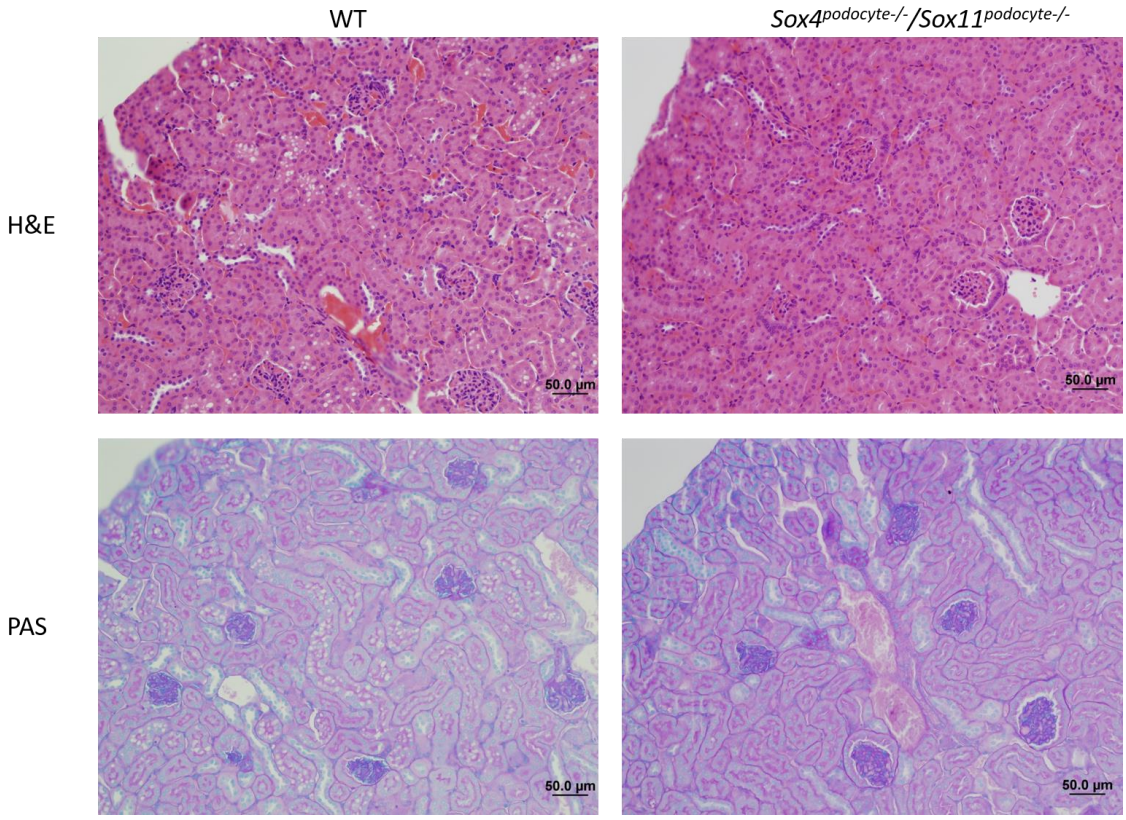

Figure 4.6. Adult *Sox4*$^{podocyte-/-}$/*Sox11*$^{podocyte-/-}$ (6 month) kidneys are indistinguishable from wild-type (WT) controls. H&E and PAS staining of the renal cortex.

previously reported when *Sox4* expression is abrogated in NPC, and that the integrity of the glomerular filtration barrier is maintained. The observed phenotype must therefore be secondary to a primary effect in a cell type derived from NPC that is not the podocyte.

4.4.3 Activation of Parietal Epithelial Cells in *Sox4*$^{nephron-}$ Mice

Over the past several decades there has been emerging interest in the study of the various resident glomerular cell types. In the 1980s and 1990s, the focus was on mesangial and endothelial cells, followed by two decades of intense study of the development and function of the podocyte. Most recently, parietal epithelial cells (PEC) have taken center stage in glomerular research (Shankland et al. 2014). PEC form the

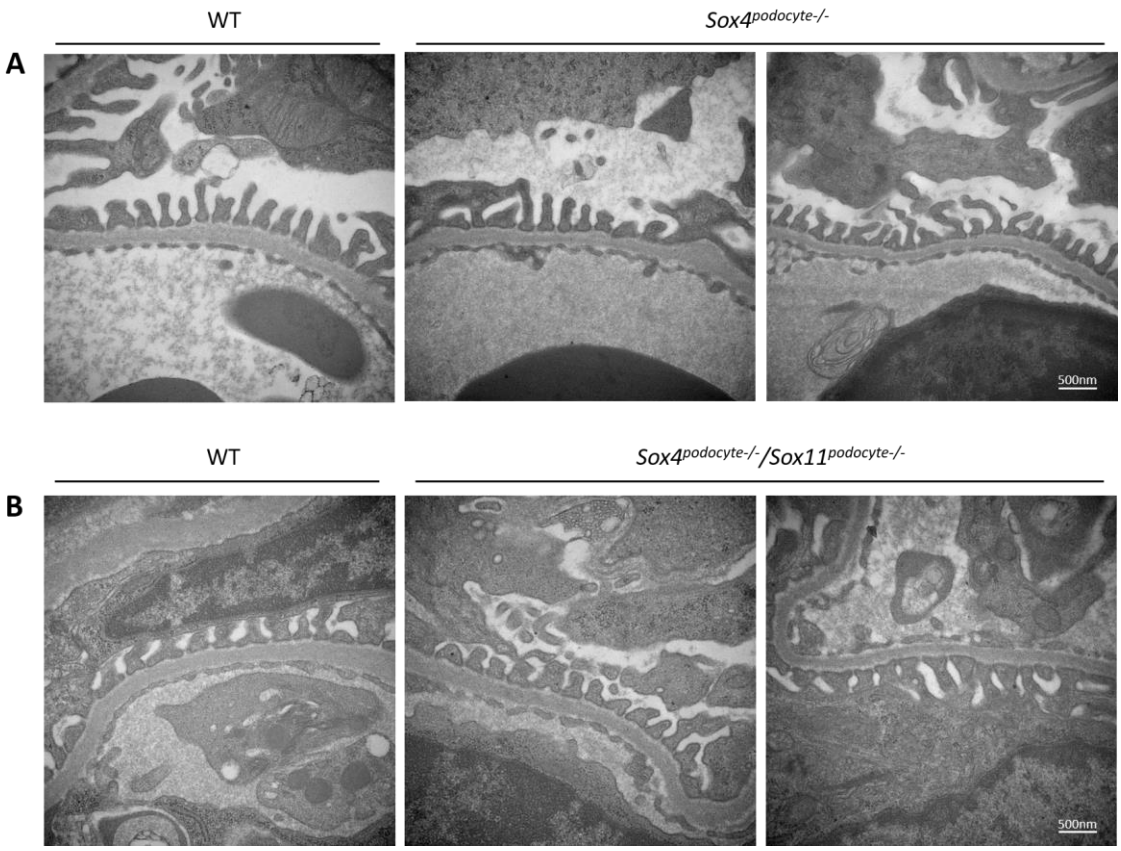

Figure 4.7. Absence of adult (6 months) ultrastructural renal phenotype in A) *Sox4*$^{podocyte-/-}$ and B) *Sox4*$^{podocyte-/-}$/*Sox11*$^{podocyte-/-}$ mice. No podocyte injury or foot process effacement can be observed and glomerular basement membrane thickness is maintained throughout the genotypes as compared to wild-type (WT).

external layer of Bowman's capsule and present with a sexual dimorphism in mice as male PEC consist of a mixture of squamous and cuboidal cells or purely cuboidal cells, whereas PEC in females present mostly as squamous cells (Ahmadizadeh et al. 1984, Yabuki et al. 1999). PEC and podocytes share a common phenotype until the S-shaped body stage, when podocytes start to lose expression of proliferation markers, whereas PEC continue to maintain those same markers (Pavenstädt et al. 2003, Ohse et al. 2009). Although multiple experiments, including metabolic labeling and lineage tracing of PEC in juvenile rats (Appel et al. 2009), have suggested that a portion of podocytes are

recruited from adjacent PEC, the hypothesis that PEC can act as podocyte progenitors remains controversial (Shankland et al. 2014).

Activated PEC (aPEC) are a subset of PEC exhibiting increased cellular activity, proliferation, relatively larger cuboidal cytoplasm, and enlarged nuclei. These cells can also exhibit increased thickness of the underlying parietal basement membrane due to increased matrix deposition (Shankland et al. 2014). Activated PEC are associated with glomerular crescents, where the proliferating PEC form a multilayered crescent that eventually will obstruct the tubular outflow, disconnect the glomerulus from its tubule and subsequently lead to glomerular degeneration and loss (Le Hir et al. 2001, Le Hir and Besse-Eschmann 2003). Progressive glomerulosclerotic lesions can also result following the adhesion of an affected capillary to Bowman's capsule by either aPEC or podocytes. The aPEC then invade the glomerular tuft and deposit extracellular matrix proteins resulting in loss of podocytes, increased mesangium and development of advanced sclerotic lesions (Figure 4.8) (Smeets et al. 2011, Smeets and Moeller 2012).

As PEC are derived from NPC (Figure 4.1), determining if the activation of PEC could be responsible for the genesis of ESKD observed in *Sox4*$^{nephron-}$ mice was explored. H&E sections from 13 month old animals were examined. Cells were observed extending from the parietal layer towards the capillary tuft (Figure 4.9C), there were several instances of multiple layers of cells (Figure 4.9D), and overall there appeared to be an increased number of cells in the parietal layer resulting in cells that appeared more tightly packed (Figure 4.9E & F) than in wild-type (Figure 4.9A & B).

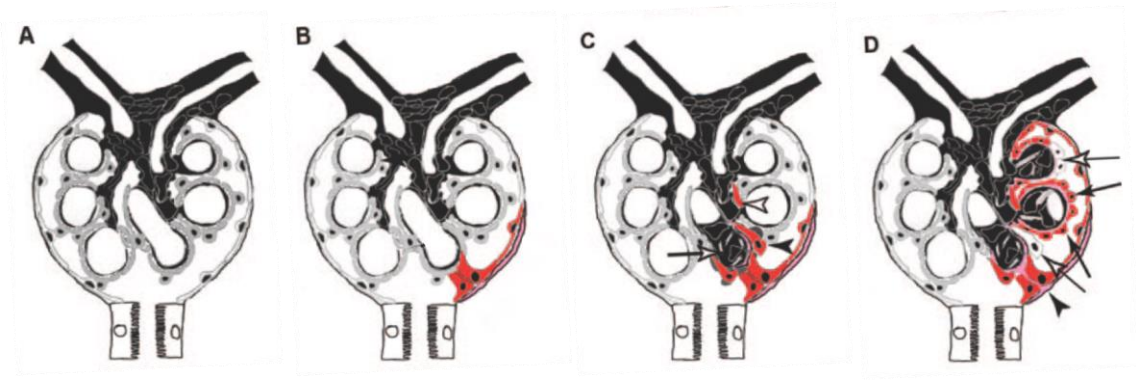

Figure 4.8. Schematic representation of progression to glomerulosclerosis by activation of PEC. A) Normal glomerulus with capillaries and mesangium (in black) surrounded by podocytes (gray cells) lining the capillary tuft and PEC (white cells) lining the inside of Bowman's capsule (BC). B) Focal activation of PEC (red) and adhesion to the capillary. Increased deposition of matrix may also occur on BC (pink matrix). C) Activated PEC invade the glomerular tuft (black arrowhead) and deposit matrix. PEC can appear disconnected from the adhesion due to sectioning (white arrowhead). Sclerosis of the glomerular tuft progresses with mesangial sclerosis developing within the affected segment (white arrow). D) Advanced sclerotic lesion in which capillary loops are covered by PEC (black arrows) and matrix (pink). Some PEC will no longer express markers of activation (white arrows). At the initial site of adhesion, a matrix bridge is maintained between BC and the glomerular tuft (black arrowhead). Adapted from Smeets et al. 2011.

In order to further confirm the activation of PEC in the absence of *Sox4* in NPC, evaluation of a greater number of glomeruli must be carried out in order to increase confidence that the observations of aPEC are not solely due to plane of sectioning. Identification of aPEC could also be carried out by staining for CD44 or claudin-1/-2, markers of aPEC (Smeets et al. 2011, Shankland et al. 2014). Evaluation of the composition of the basement membrane could also serve to indicate PEC activation. The GBM, not unlike other basement membranes, is principally composed of networks of laminin, type IV collagen, nidogens and heparin sulfate proteoglycans. The isoforms of both laminin and type IV collagen in the GBM are known to change with the

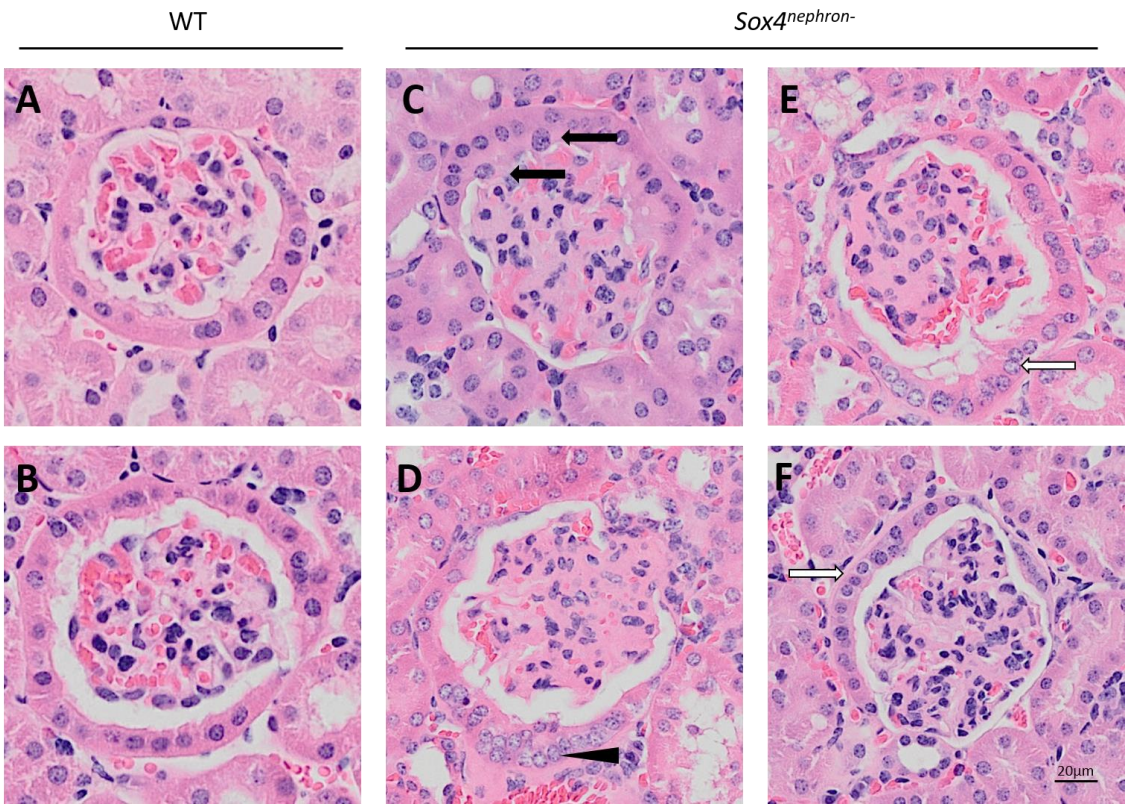

Figure 4.9. Activation of parietal epithelial cells in 13 month old *Sox4*$^{nephron-}$ mice. Various phenotypic manifestations of activation are observed much more frequently in *Sox4*$^{nephron-}$ mice(C-F) than in wild-type (WT) controls (A-B). Black arrows – cells extending (migrating) towards capillary tuft. Arrow head – multiple layers of parietal cells. White arrows – increased number of parietal cells, more tightly packed than in WT.

development and maturation of the glomeruli. Type IV collagen is composed of triple helical heterotrimers. Six distinct collagen IV α chains form only three different heterotrimers: α1α2α1(IV), α3α4α5(IV), α5α6α5(IV). The α1α2α1(IV) synthesized by the endothelial cells and the podocytes of the immature GBM is replaced by α3α4α5(IV), synthesized solely by the podocytes in the mature GBM (Abrahamson et al. 2009). α5α6α5(IV) is not detected in the GBM under normal conditions, however it is a major component of the basement membrane of Bowman's capsule (Ninomiya et al. 1995), and aPEC have also been shown to deposit "Bowman's capsule type matrix" including α1α2α1(IV) atop of the existing GBM (Cai et al. 1996, Smeets et al. 2011).

Thus an analysis of the composition of the GBM thickening observed in *Sox4*$^{nephron-}$ mice could help to ascertain the cellular origin of the increased matrix. Further investigation with an additional conditional knockout approach targeting PEC, would also increase our understanding of the role of *Sox4* in the glomerulus. This could be done using the Pax8Cre which targets both the visceral and parietal epithelial cells of maturing glomeruli from the late S-shaped body stage as well as UB cells (Bouchard et al. 2004, Grouls et al. 2012). Taken together, with data from our *Sox4*$^{nephron-}$ mice, such animals would allow us to better tease out the cellular and molecular mechanisms underlying the role of *Sox4* in kidney development and maintenance.

In summary, a reduction in cell cycling of NPC was identified as the probable source of reduced PTA and subsequent reduced nephron endowment observed in the absence of *Sox4* in NPC. *Sox4* and *Sox11* alone and in combination are dispensable for proper podocyte differentiation and function. The absence of Sox4 expression in podocytes does not recapitulate the loss of integrity of the glomerular filtration barrier observed in *Sox4*$^{nephron-}$ mice. Histological observations suggest that an activation of PEC is present in *Sox4*$^{nephron-}$ mice and could, along with the increased stress due to significant reduction in glomerular number, potentiate the genesis of the early-onset proteinaceous glomerular injury as well as its progression. Future studies will properly assess the activation status of PEC and conditional gene deletion studies will be aimed at recapitulating the phenotype observed in *Sox4*$^{nephron-}$ mice. Additionally, gene deletion studies are needed to evaluate the independent role of *Sox11*, as well as the combined role of *Sox4/Sox11*, signalling during renal development *in vivo*.

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5. Conclusions and future directions

5.1 Summary of Major Findings

A descriptive analysis of the expression of *Sox4* in the developing kidney was first performed. The expression of *Sox4* is highest in the early stages of developing kidney, and lowest in the adult. *Sox4* is expressed in nephron progenitor cells (NPC) and ureteric bud (UB) and continues to be expressed in differentiated nephrogenic structures just prior to birth (E18.5) as detected by *in situ* hybridization.

Because *Sox4* knockout mice die at E14, prior to the completion of nephrogenesis, I have employed a conditional knockout approach, using a floxed *Sox4* allele (Penzo-Méndez et al. 2007) and Six2-Cre (Kobayashi et al. 2008), in order to study the role of *Sox4* in kidney development. Conditional ablation of *Sox4* in the nephrogenic lineage ($Sox4^{nephron-}$) results in post-natal glomerular disease *in vivo* characterized by tubular dilatation, poorly defined glomerular tufts, reduced cellularity of the renal corpuscle and disorganization of the filtration barrier, which ultimately leads to end stage renal failure by 5 to 9 months of age and death of the animal (Chapter 2). As early as 2 weeks after birth, kidneys exhibit numerous periodic acid-Schiff (PAS) positive protein casts in dilated tubules. Renal corpuscles were hypocellular and had poorly defined glomerular tufts, with an increase in mesangial matrix. These mice also exhibited a focal loss of the proximal tubular brush border. A closer look at the ultrastructure of the glomerular filtration barrier revealed that in the absence of *Sox4*, glomeruli display extensive podocyte foot process effacement and a thickening of the glomerular basement membrane (GBM). By P7 glomeruli also exhibited a reduction in the slit diaphragm protein nephrin and a reduction in the number of Wt1+ podocyte

nuclei suggesting that *Sox4* may play a role in podocyte development and maintenance. Further *Sox4*$^{nephron-}$ mice exhibit a reduction in nephron number. This was first observed anecdotally as the histological slides were examined and then quantified using the acid maceration technique. I was able to accurately confirm this fact both embryonically and post-natally by adapting the gold standard physical disector/fractionator method for glomerular quantification. In an effort to understand the origin of reduced nephron number, explant experiments were carried out on embryonic kidneys and revealed that the reduced nephron endowment in *Sox4*$^{nephron-}$ mice results from the formation of fewer pretubular aggregates (PTA). I further sought to investigate if the formation of fewer PTA resulted from increased apoptosis, decreased proliferation or a change in cell fate of NPC. Preliminary data indicate no changes in apoptosis and a decrease in cell proliferation of NPC in *Sox4*$^{nephron-}$ kidneys.

Research continues to further elucidate the molecular mechanisms involved in kidney development, and many of the steps required to build a kidney have been identified. However, in a more general sense, what determines if a human kidney will develop 300,000 or 1,000,000 nephrons has not been identified. Although the precise molecular mechanisms of the observed phenotypes require further investigation, I have presented here definitive evidence that *Sox4* is essential for proper nephron endowment. I have also established the integrated disector method as an updated adaptation of the gold standard physical disector/fractionator method for unbiased estimation of glomerular number (Chapter 3).

A reduction in nephron number alone would be insufficient to explain the severity of the

phenotype observed in *Sox4*$^{nephron-}$ mice. To further pursue the hypothesis that *Sox4* is essential in podocyte development and maintenance I generated a conditional knockout strain of *Sox4* in podocytes (*Sox4*$^{podocyte-/-}$). The absence of *Sox4* expression in podocytes does not recapitulate the *Sox4*$^{nephron-}$ phenotype and the integrity of the glomerular filtration barrier is maintained. The observed phenotype in *Sox4*$^{nephron-}$ mice must therefore be secondary to a primary effect in a cell type derived from NPC that is not the podocyte. Finally, I have presented preliminary data to suggest a second role for *Sox4* in the kidney as a potential repressor of parietal epithelial cell activation (Chapter 4).

5.2 Future Directions

The research presented herein clearly demonstrates that the null hypothesis "*Sox4* is not required for normal murine kidney development *in vivo*" must be rejected. Although much progress has been made into understanding the role of *Sox4* in kidney development, as with most research, more questions than answers remain. Multiple approaches were used to address the role of *Sox4* in kidney development, not all of them presented in detail in this thesis. Multiple compartments and cell types were targeted using the conditional knockout approach (APPENDIX E, F). For various reasons not all models were studied in detail. A summary table of mouse models evaluated over the course of the presented thesis and studied by the author can be found in APPENDIX G. Because *Sox4* knockout mice die prior to the completion of nephrogenesis, observations were also made with *Sox4*$^{-/-}$ embryonic kidneys at E13.5 and E14.5 just prior to embryonic death. An increased prevalence of glomerular dilatation compared to wild-type controls was observed at E14.5 (APPENDIX H). To further the study of *Sox4* in the kidney, other simple experiments using *Sox4*$^{-/-}$ embryonic kidneys could help complete the story. If explant

experiments with *Sox4*$^{-/-}$ kidneys recapitulate the decrease in number of PTA observed, this would increase the strength of the observation that the primary role is in NPC. However, a clear indication of the role *Sox4* is playing would be more difficult to interpret in this system as *Sox4* expression would be absent in all lineages. A more severe phenotype could indicate complementary roles in cross signaling with the UB and other cell types. While a less severe phenotype could indicate that *Sox4* is playing an opposing role in the UB, such as to reduce signaling from the UB to NPC.

Observations in *Sox4*$^{-/-}$ embryos also led me to discover an elongated gonad phenotype (APPENDIX I). This phenotype was also observed in the Pax3Cre conditional knockout of *Sox4* (data not shown). Later, in collaboration with Dr. Peter Koopman's group, I was able to confirm this observation in the *Sox4*$^{-/-}$ embryos (APPENDIX J).

Reduction in nephron number in the absence of *Sox4* in NPC may be the first insult leading to the end-stage kidney disease (ESKD) phenotype. A compensatory hyperfiltration and hypertrophy of the nephrons can result in increased stress of the glomerular environment and heightened susceptibility to injury and insult. I was not able to definitively identify the cell type causing primary injury, however it is clear from the data presented herein that *Sox4* is dispensable in podocytes. The majority of glomerular diseases can be associated with segmental or global glomerular scarring, regardless of which glomerular cell type is primarily injured (Shankland et al. 2014). Local podocyte damage can also spread to induce injury in otherwise healthy podocytes and further affect glomerular endothelial and mesangial cells (Ichikawa et al. 2005).

It is also possible that the primary role of *Sox4* is in the maintenance of the proximal

tubule. Sustained injury to the proximal tubule can also result in interstitial fibrosis. Others have shown that this is then followed by capillary loss and glomerulosclerosis as well as distal tubule injury, indicating a cross talk between proximal and distal tubule as well as between proximal tubules and fibroblasts (Grgic et al. 2012, Takaori et al. 2016). It has also been shown that proximal tubule injury leads to cell cycle arrest of epithelial cells and activation of signaling pathways that activate pro-fibrotic gene transcription, resulting in vascular rarefaction, myofibroblast proliferation and extra-cellular matrix deposition in the interstitium (Bonventre 2014a, 2014b). Prolonged stress and injury to tubular epithelial cells, can result in the release of circulating factors via inflammatory cells which can lead to podocyte detachment, effacement and apoptosis (Leung et al. 2014). This should also be further investigated. Generally speaking, glomerular injury is usually seen before tubular injury. It is albeit, tubular injury and increased protein trafficking across tubules that results in the irreversible tubulointerstitial fibrosis that ultimately leads to ESKD (Leung et al. 2014)

The Sox genes are transcription factors and as such, a primary mechanism of action of SoxC in the developing kidney will be to modulate the expression of other genes. The Sox family of genes all contain a highly conserved high-mobility group (HMG) domain which binds the minor groove of DNA and recognizes the consensus sequence A/TA/TCAAA/TG (Wilson and Koopman 2002). In order to confer specificity to gene modulation by Sox genes, interaction with protein partners are required (Kamachi et al. 2000). This model explains how, based on protein partner pairing, the same SOX protein can differentially affect gene expression in various cell types, as well as in the same cell type at different stages. Sequences recognized by the HMG domain of the

SOX proteins, in combination with other sequences recognized by interacting protein partners, confer larger specificity to target sequences and modulation of specific genes (Kondoh and Kamachi 2010). Identification of SOXC interacting protein partners will be key to understanding the role they play in the developing kidney. Identification of SOX4 target genes through techniques such as RNA-Seq will also be crucial in further elucidating the mechanism of action of *Sox4* in the kidney. The continued identification and understanding of fundamental processes which underlie the development of a functioning kidney is essential. Herein I have presented my contribution to our understanding of kidney development in hopes that some day it may further our understanding of the etiology of kidney disease and potentially to the identification of new therapeutic targets.

5.2 References

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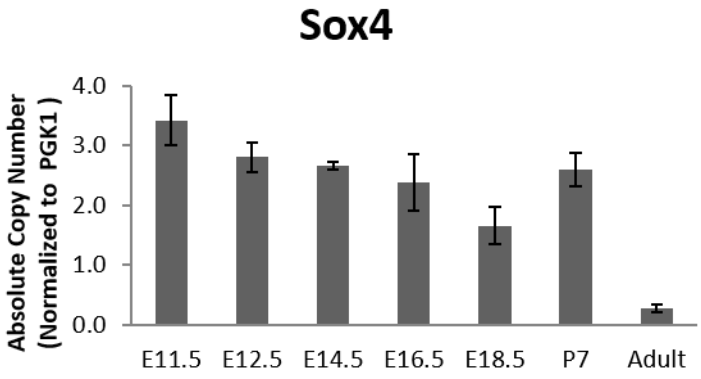
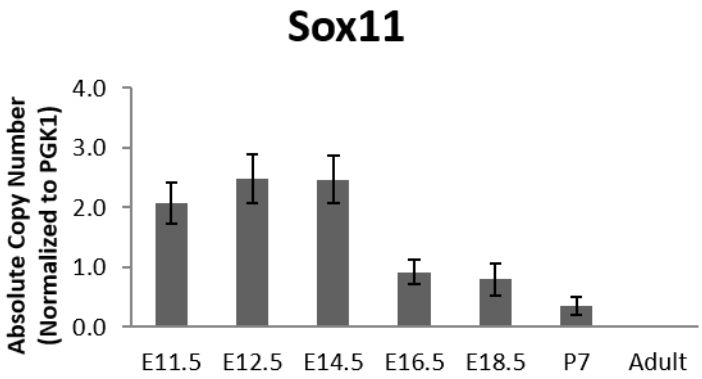
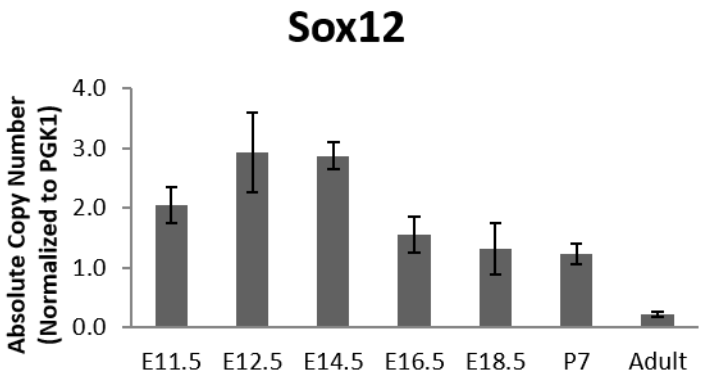
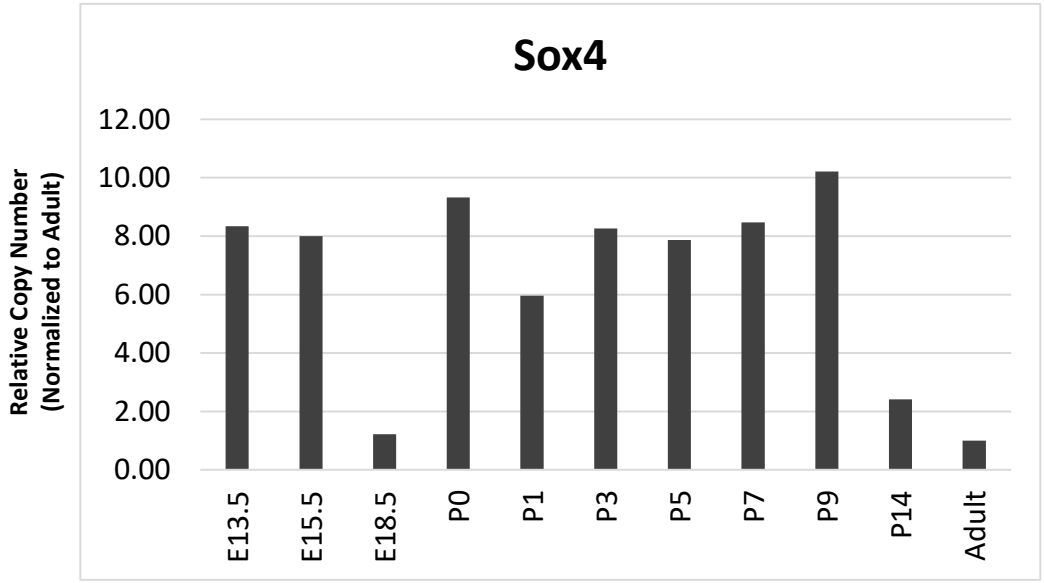
APPENDIX A – Absolute copy number for each SoxC gene. Copy number for each gene was normalized to the absolute copy number of Pgk1 for each sample.







APPENDIX B – Expression of *Sox4* remains high postnatally. Absolute copy number of *Sox4* gene expression was established and normalized to absolute copy number in the adult. Although a reduction in expression is observed at embryonic day (E)18.5, expression remains at levels similar to those observed in early embryonic time points after birth before a significant reduction is observed between postnatal day (P)9 and P14.



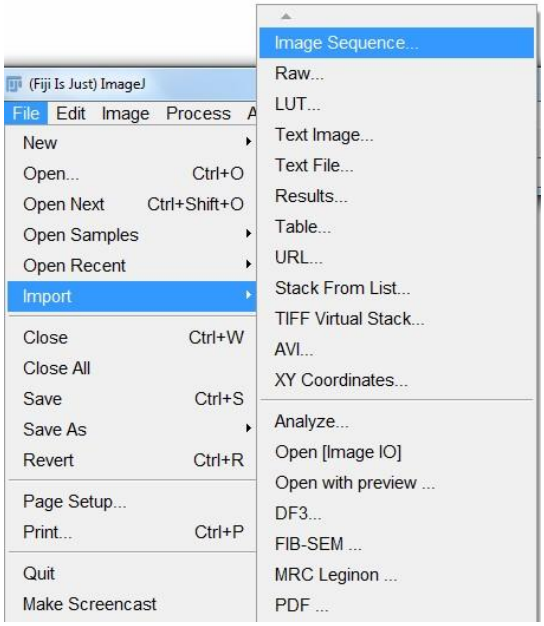
APPENDIX C - Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) technical information.

Sample/Template	Details
Source	Materials and Methods: Kidneys from CD1 mice
Method of preservation	Materials and Methods: RNAlater (Ambion, Austin, TX)
Storage time (if appropriate)	Materials and Methods: Tissues were stored at -80°C for < 2 months. RNA was stored at -80°C and reverse transcribed within two weeks. cDNA was stored at -40°C and analyzed within 3 months.
Handling	Frozen, see "Storage Time".
Extraction method	Materials and Methods: RNAqueous-4PCR Kit (Ambion)
RNA: DNA-free	DNAse step using DNA-*free* (Ambion) in preparation protocol. For each cDNA sample a "Minus RT Control" was also prepared.
Concentration	Concentration of all RNA preparations were quantified using a Nanodrop 1000 (ThermoScientific).
RNA: integrity	Integrity of all RNA preparations were assessed by gel electrophoresis and spectral absorption ratios.
Inhibition-free	A representative set of 7 samples was used to test for the absence of inhibitors by dilution series.
Assay optimisation/validation	
Accession number	Sox4 (NM_009238.2), Sox11 (NM_009234.6), Sox12 (NM_011438.2)
Amplicon details	Amplicon sizes: Sox4 (106bp), Sox11 (129bp), Sox12 (82bp).
Primer sequence	Materials and Methods
*Probe sequence**	N/A
In silico	Specificity of all primers were validated by BLAST analysis
empirical	Materials and Methods: All primers were used at a concentration of 300nM and annealing temperatures were 57°C or 60°C. PCR product specificity was determined by melt curve analysis following each run using a heating program from 72°C to 95°C in 1 degree steps with a 5 second hold per step.

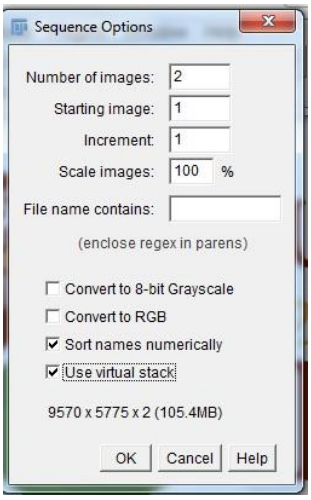
Priming conditions	For cDNA synthesis oligo-dT primers were used as provided with the SuperScript III First-Strand System (Invitrogen).
PCR efficiency	Materials and Methods: Efficiencies were determined using dilution series of kidney cDNA. Sox4 Efficiency = 0.96, R^2 = 0.998; Sox11 Efficiency = 0.99, R^2 = 0.999; Sox12 Efficiency = 0.93, R^2 = 0.999.
Linear dynamic range	Spanning unknown targets as determined by dilution curves of cDNA as well as 7 samples from 3 x 10^6 to 300 copies of plasmid DNA for *Sox4*, *Sox11*, *Sox12*
Limits of detection	LODs were not determined in this study.
Intra-assay variation	Intra-assay variations of technical triplicates for all samples resulted in a Ct standard deviation <0.16 cycles
RT/PCR	
Protocols	Materials and Methods. Reactions were prepared with 5µL of cDNA, 300nM of each primer and PerfeCta SYBR Green FastMix (Quanta Biosciences) in a final volume of 25µL and run in triplicate. Cycling conditions were 95°C for 15 minutes; 46 cycles of 95°C for 15 seconds (s), 57°C (*Sox11*) or 60°C (*Sox4* and *Sox12*) for 30 s, and 72°C for 30 s.
Reagents	Materials and Methods.
Duplicate RT	The RT step was not performed in duplicate in this study.
NTC	A NTC sample was included with each run. No amplification was observed in all but two runs, in which Ct for NTCs were detected >10 cycles after any sample.
NAC	N/A
Positive control	Plasmid DNA samples were used as inter-run calibrators.
Data analysis	
Specialist software	Data was analysed using RotorGene6 software (Corbett Research, Sydney, Australia)
Statistical justification	N/A
Transparent, validated normalisation	N/A

APPENDIX D – General steps for disector counts using TrakEM2

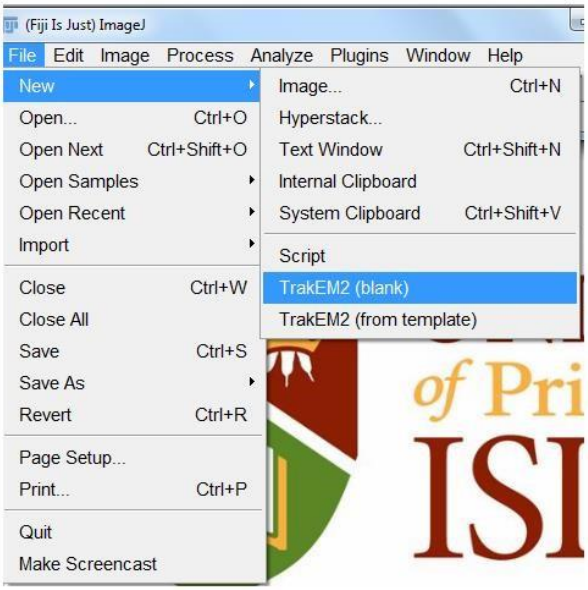
1. Import images as a single stack
    a. File> Import > Image Sequence…



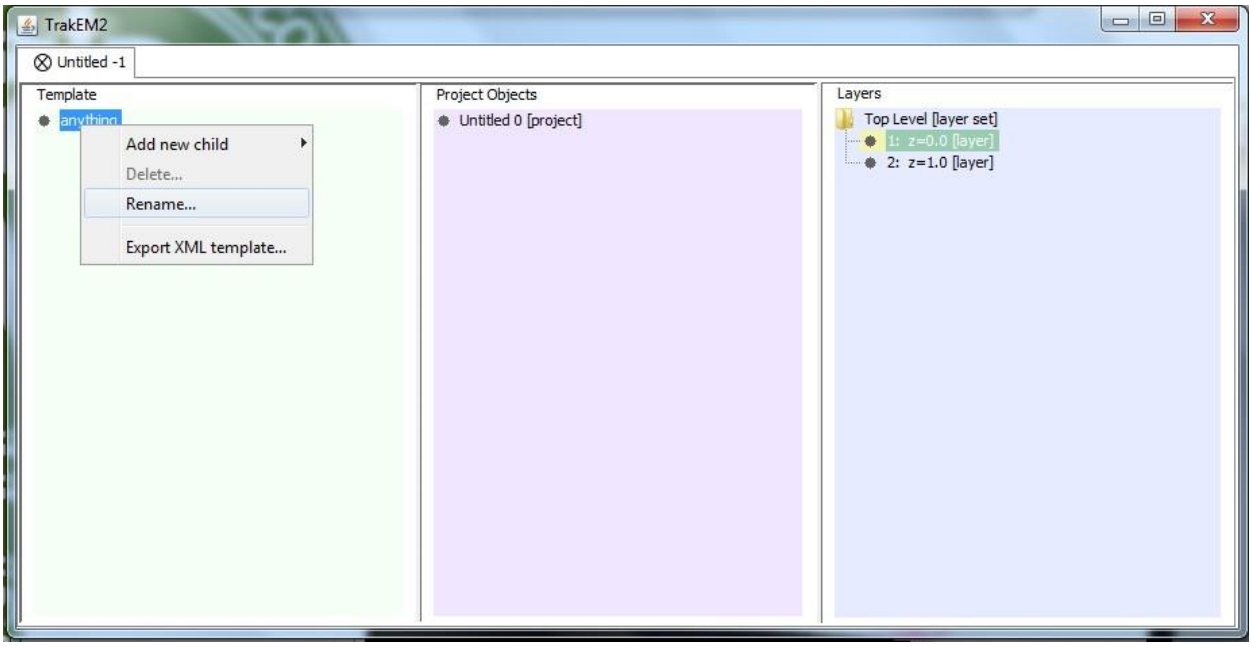
    b. Select folder with reference and lookup sequence and select “Use virtual Stack” in order to save on memory.



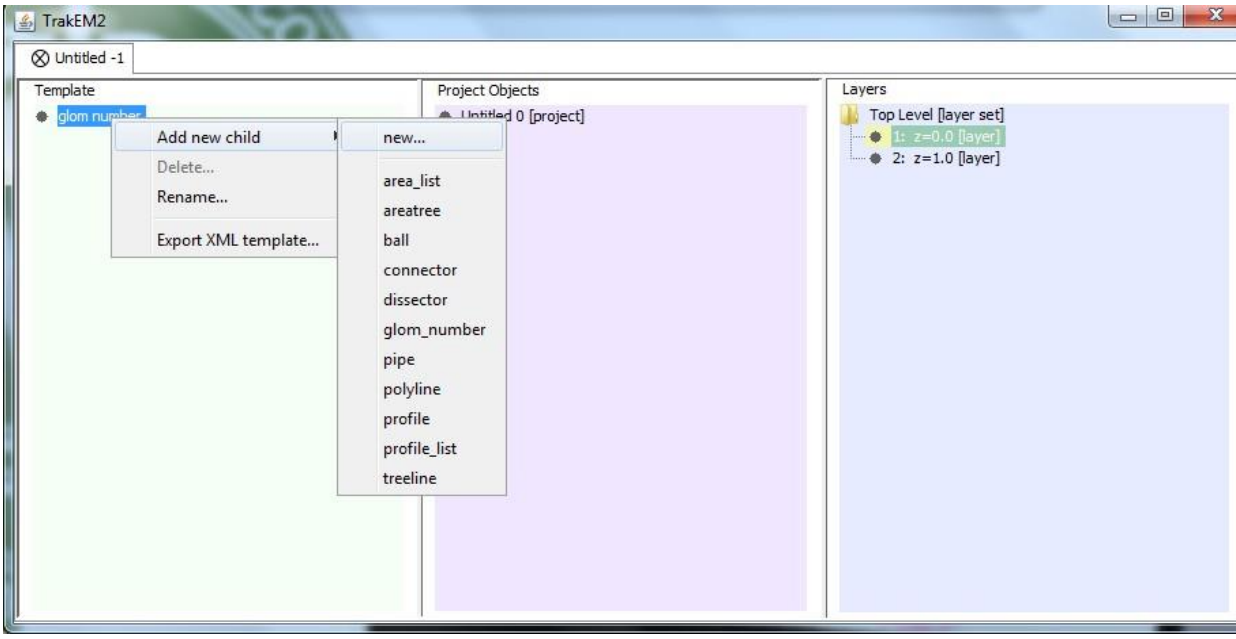
2. Open TrakEM2 environment
    a. File> New> TrakEM2 (blank)
        *later a template can be created to save some time and the TrakEM2 (from template) option can be used *



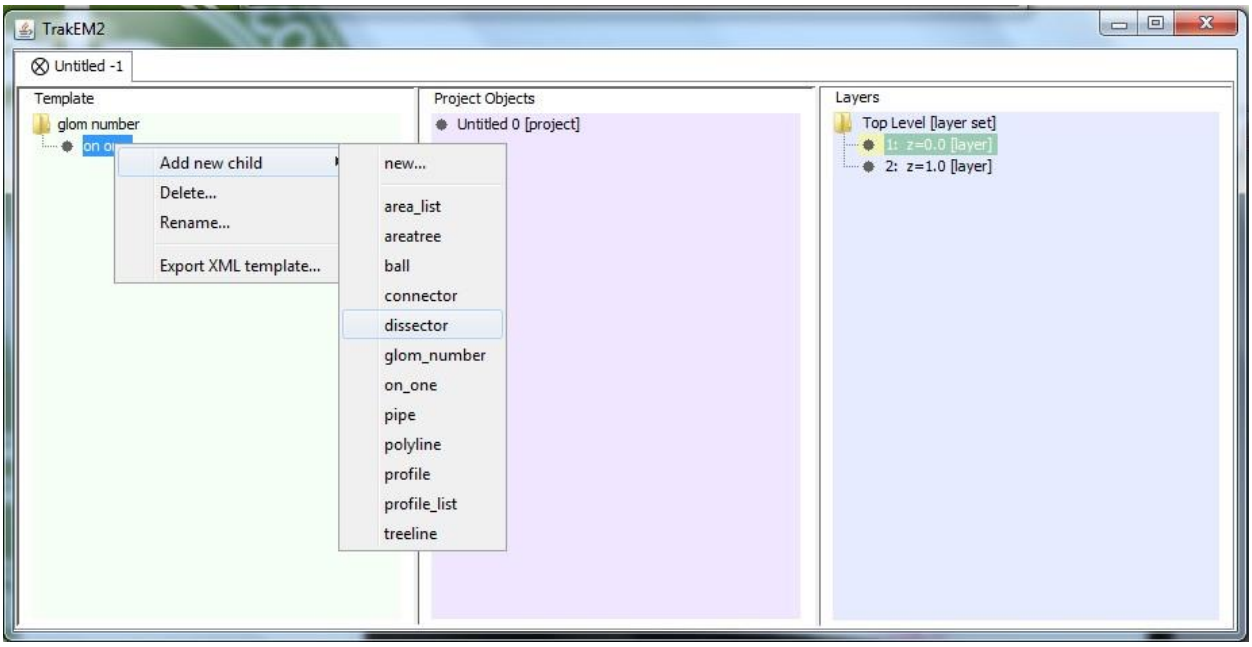
3. Set up the Template and Project Objects (dissector, area_lists, etc.)
    a. Create a template with the items to be used in the TrakEM2 project



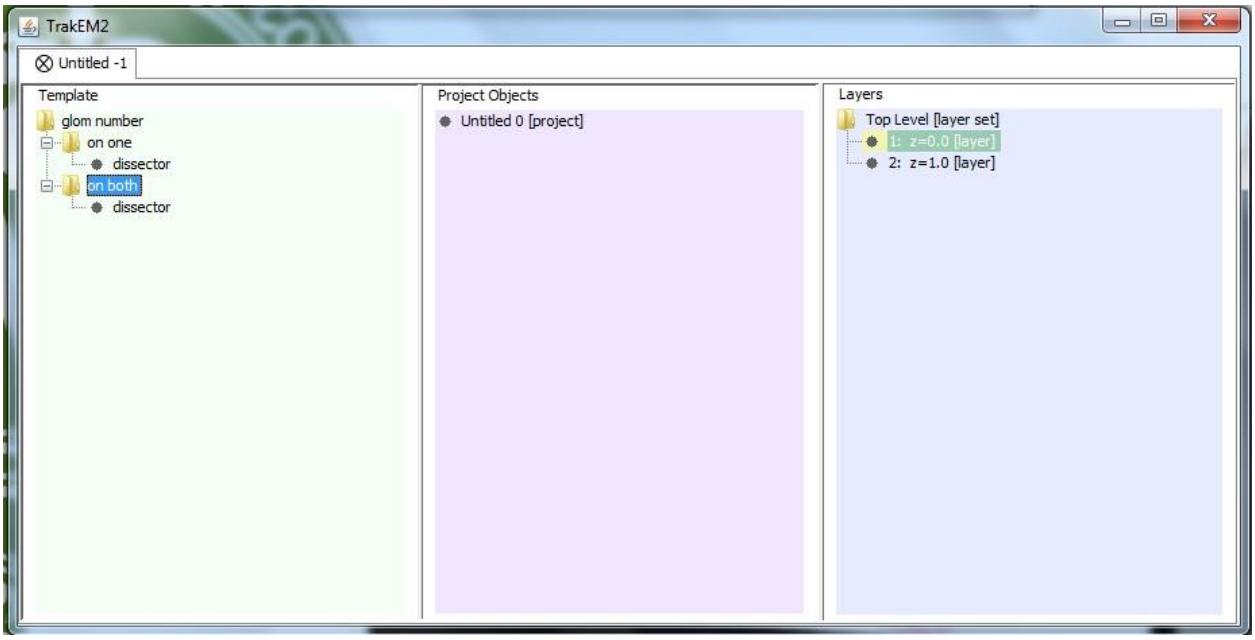
    b. Create child nodes for each of the object categories

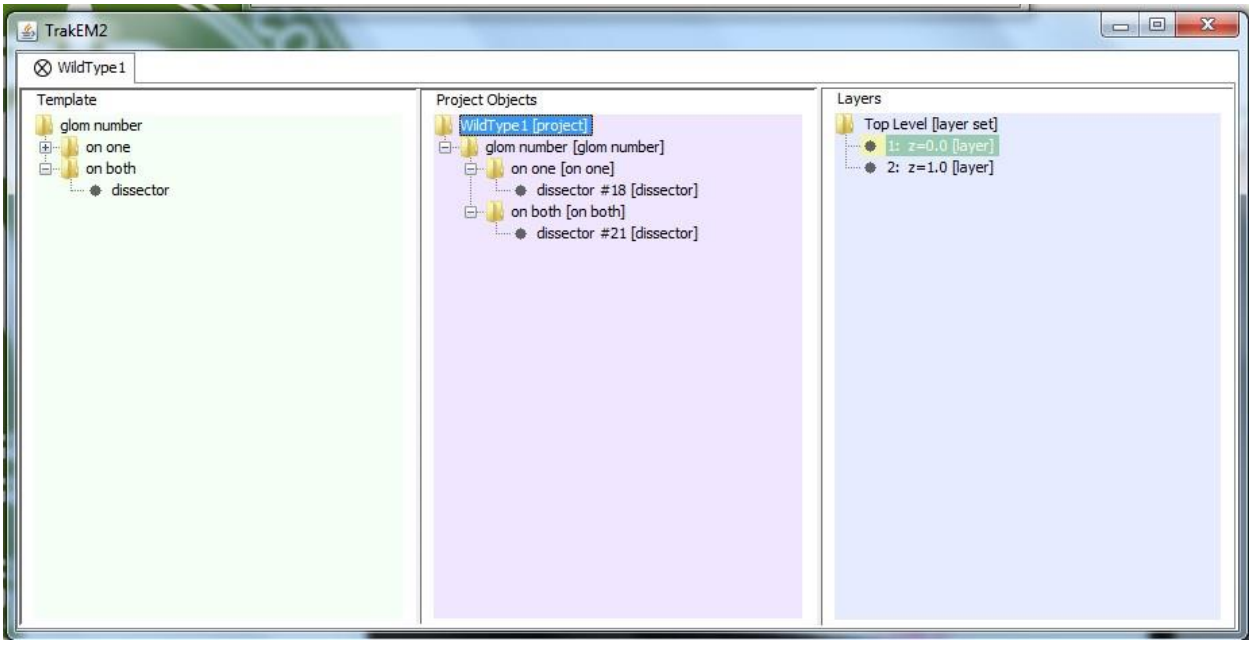


c. Add a disector to each object category

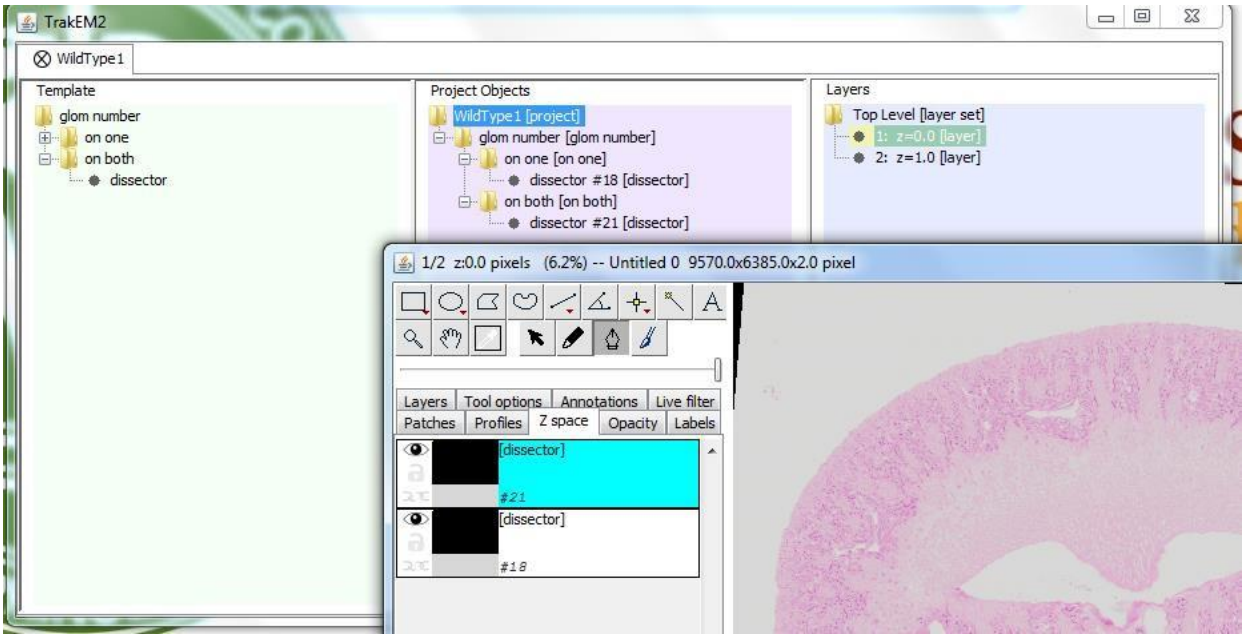


d. Drag and drop items from the “Template” area to the “Project Objects” area





e. Disectors can be renamed to distinguish them in the TrakEM2 environment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Project Objects
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glom number [glom number]
on one [on one]
dissector #18 [dissector]
on both [on both]
dissector #21 [dissector]
Layers
Top Level [layer set]
1: z=0.0 [layer]
2: z=1.0 [layer]
1/2 z:0.0 pixels (6.2%) -- Untitled 0 9570.0x6385.0x2.0 pixel
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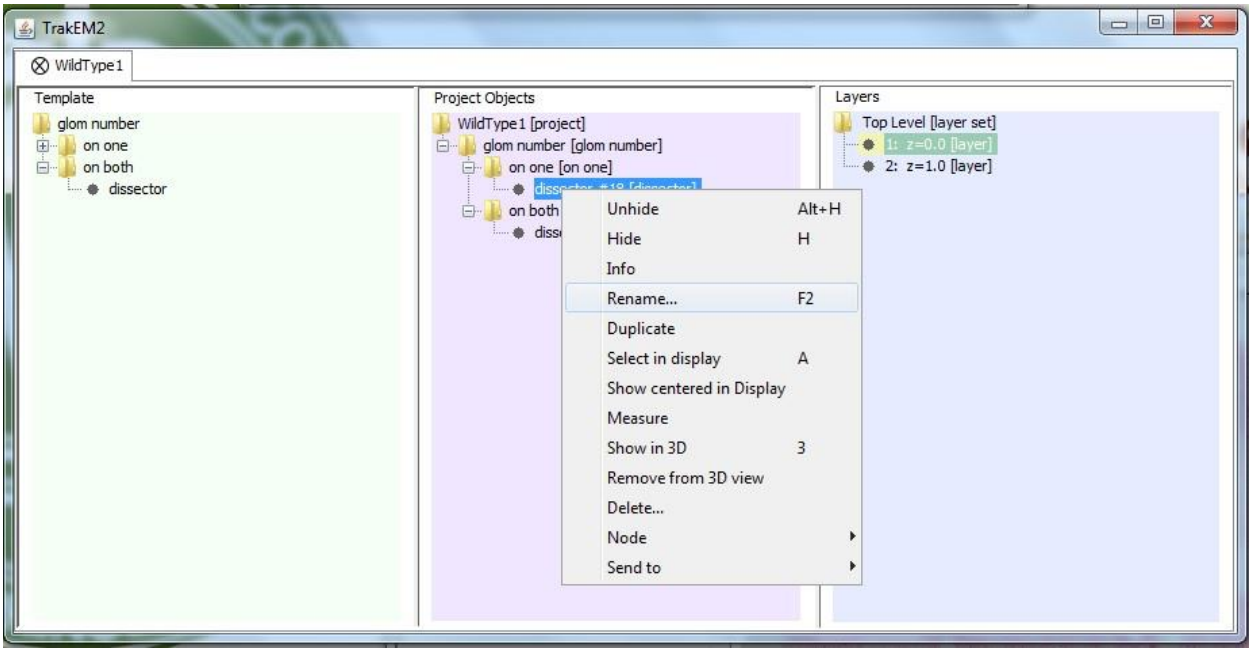
Annotations
Live filter
Patches
Profiles
Z space
Opacity
Labels
[dissector]
#21
[dissector]
#18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2: z=1.0 [layer]
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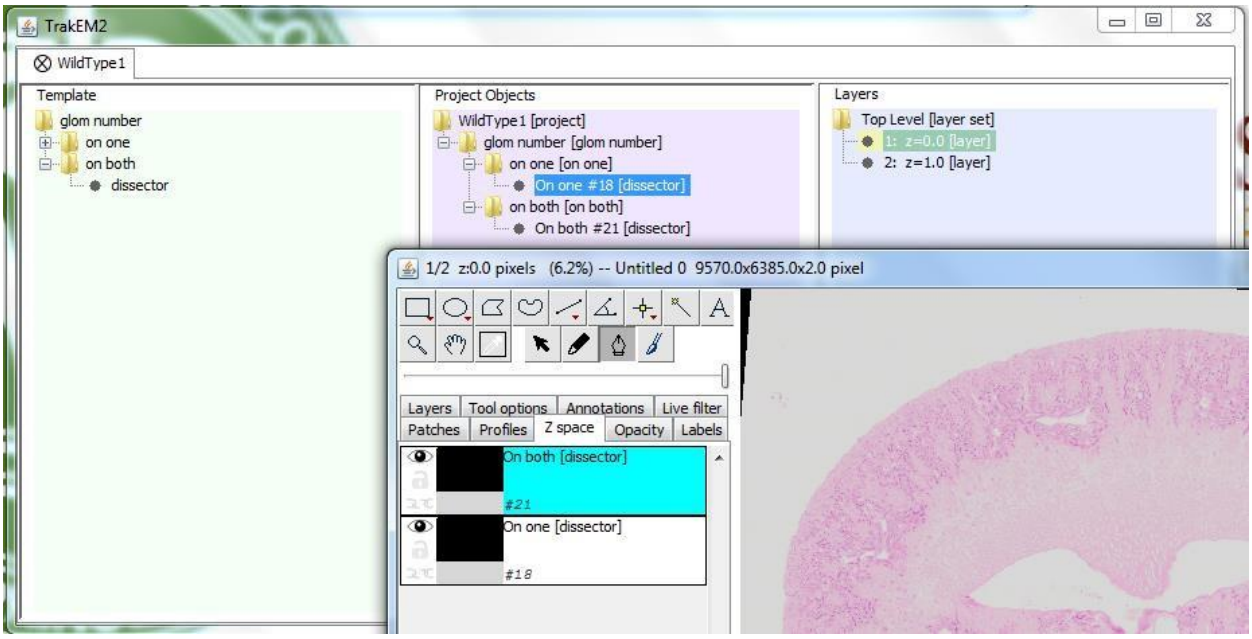
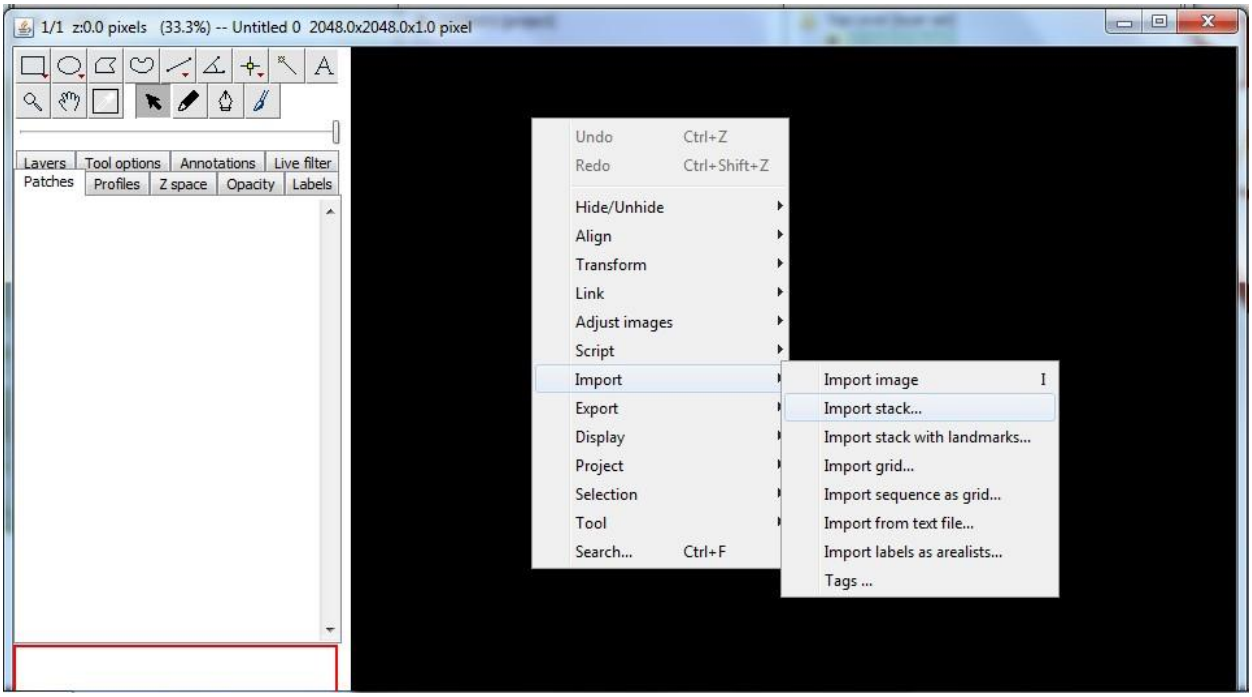
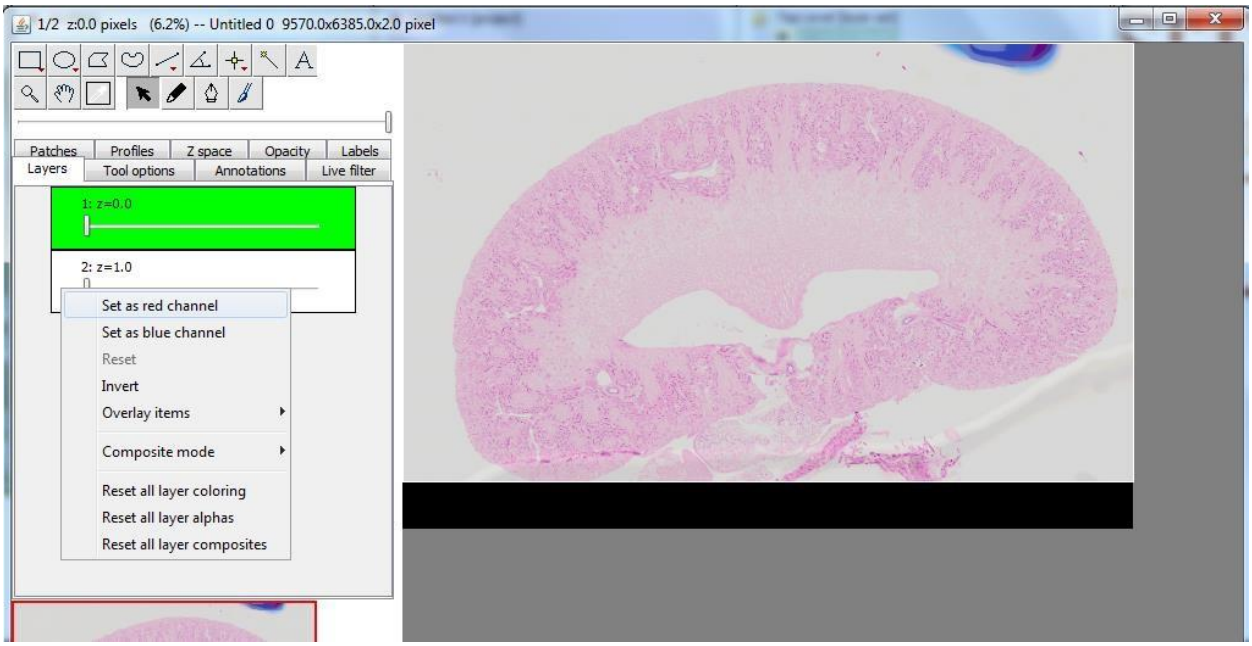


4. Import the image sequence into TrakEM2



5. Align the layers manually or automatically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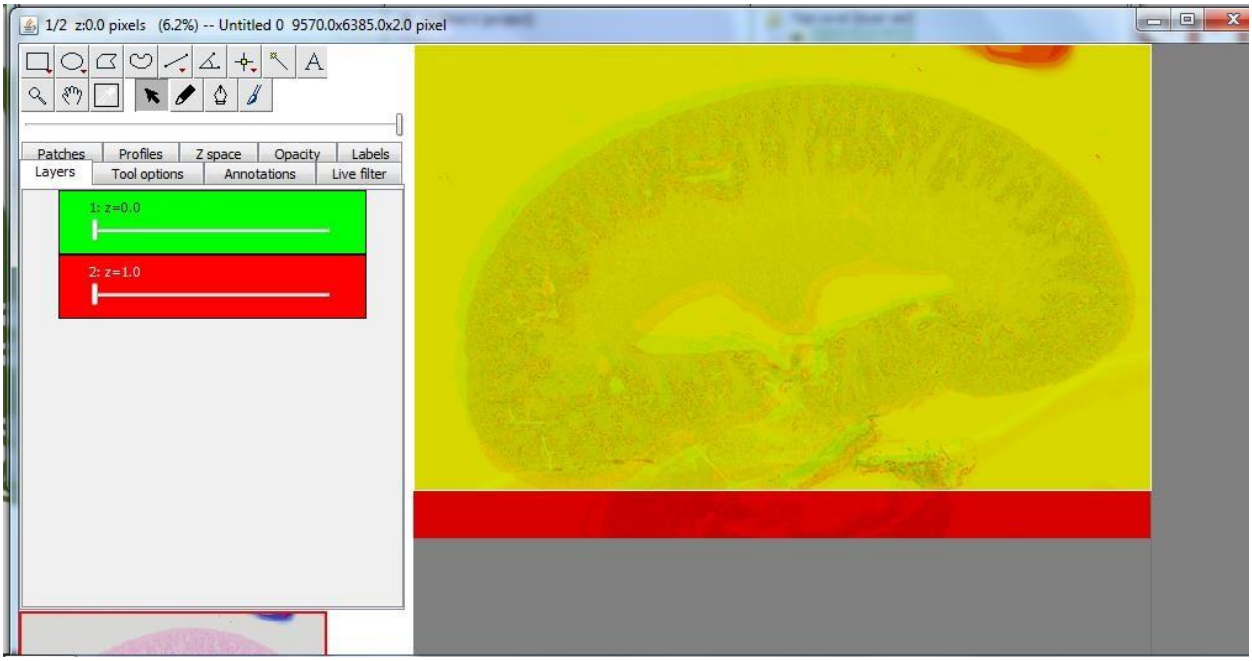
Reset all layer composites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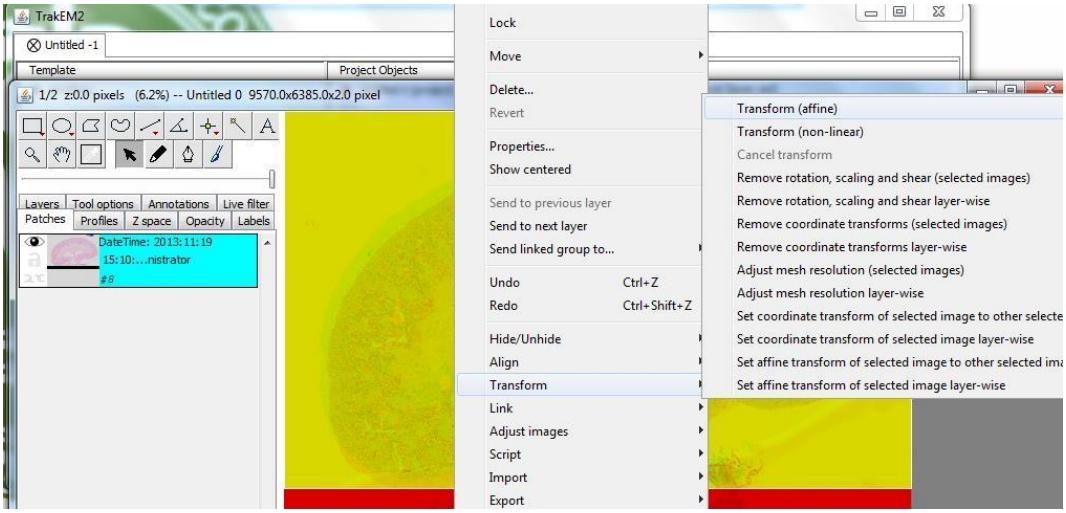
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Align
Transform
Link
Adjust images
Script
Import
Export
Transform (affine)
Transform (non-linear)
Cancel transform
Remove rotation, scaling and shear (selected images)
Remove rotation, scaling and shear layer-wise
Remove coordinate transforms (selected images)
Remove coordinate transforms layer-wise
Adjust mesh resolution (selected images)
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Set coordinate transform of selected image layer-wise
Set affine transform of selected image to other selected ima
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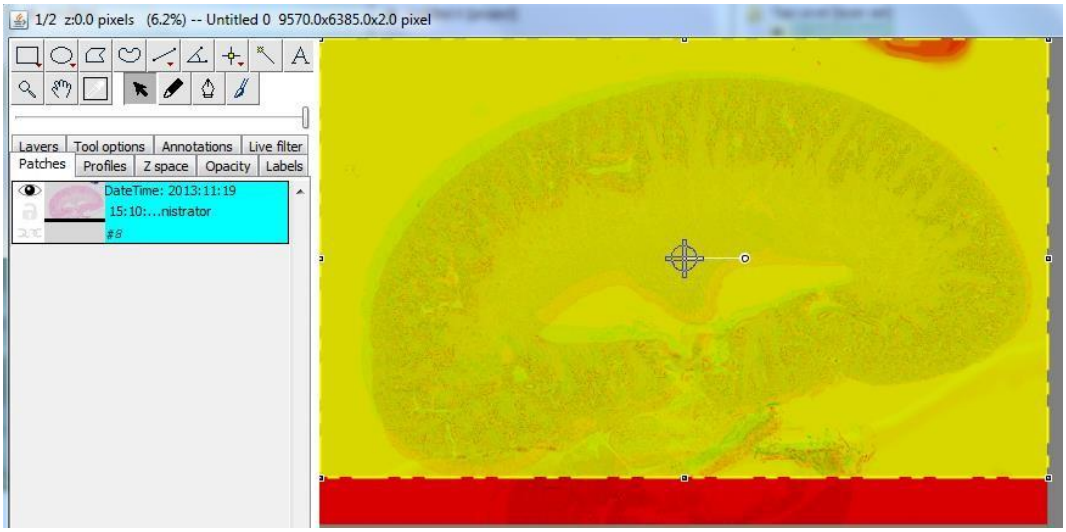
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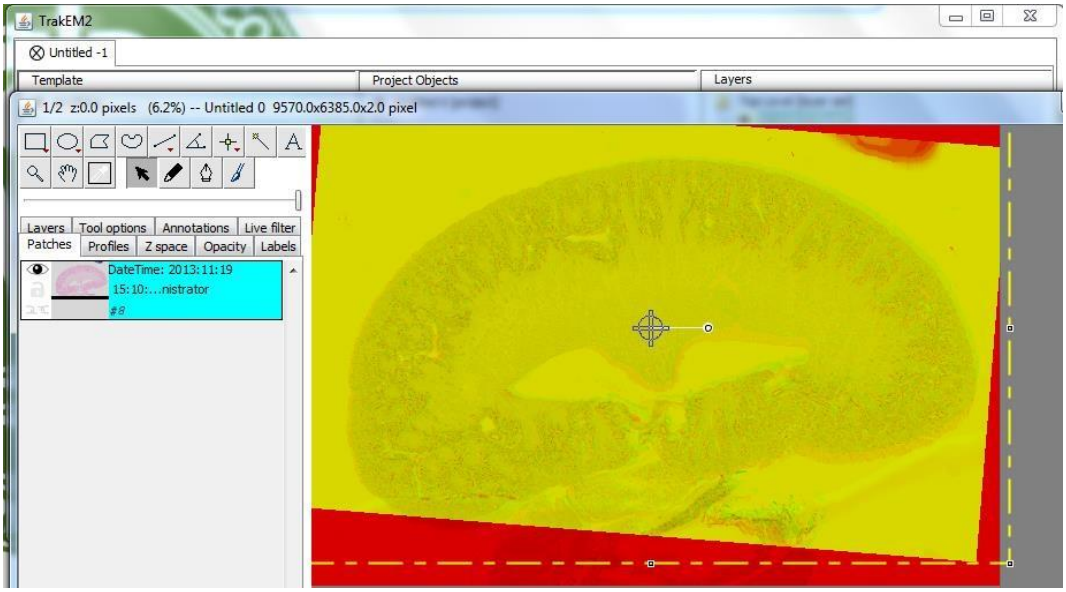
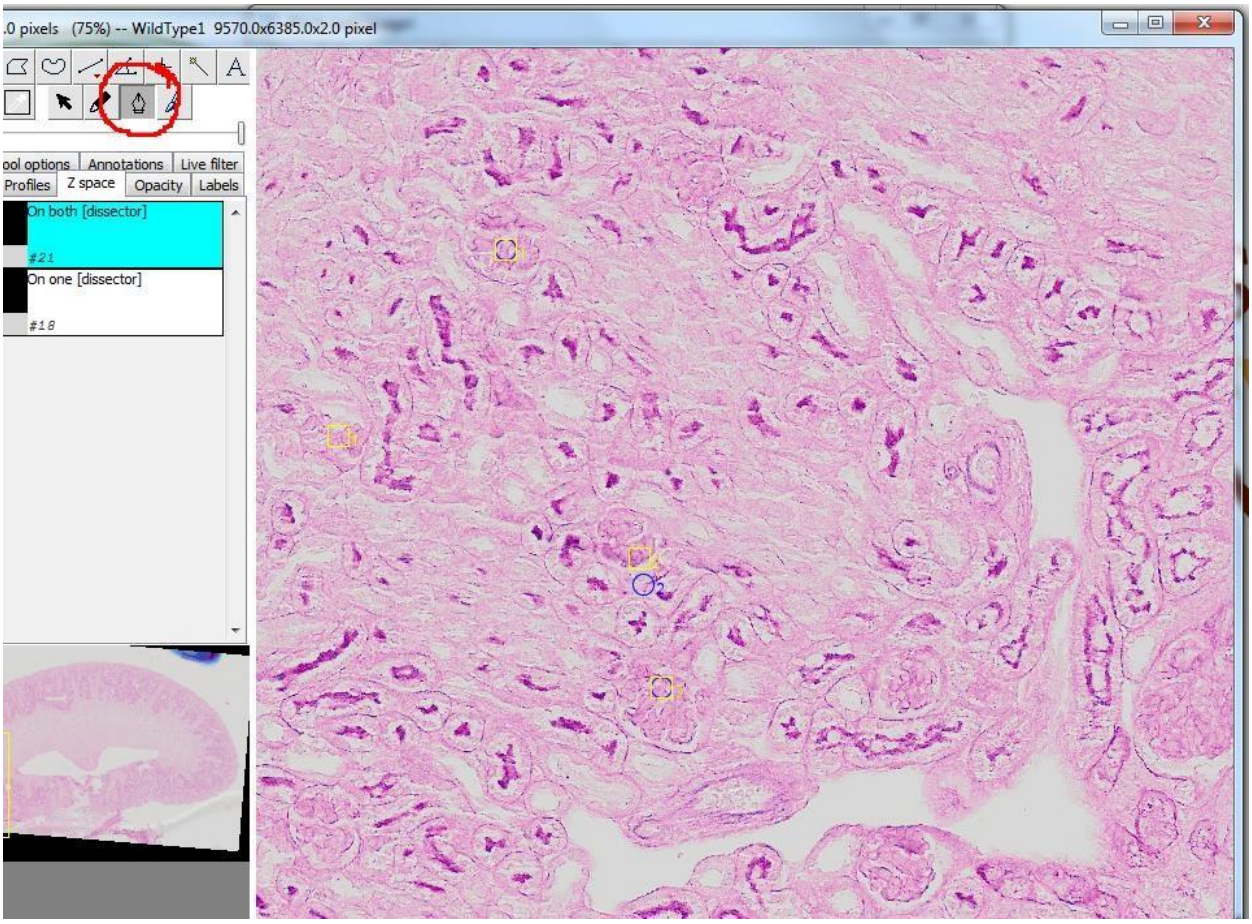
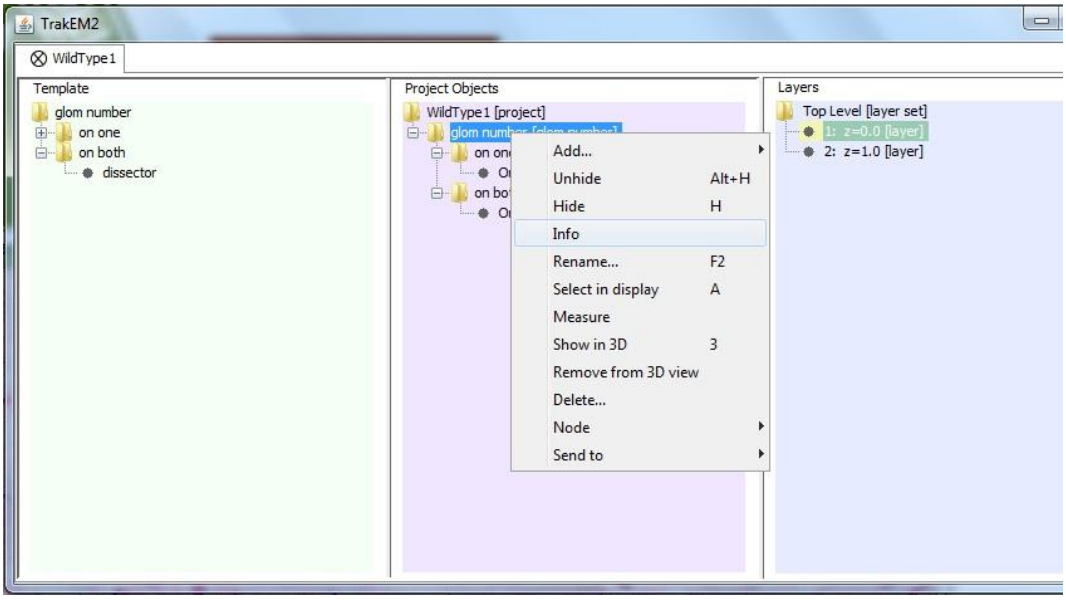


6. Identify disector particles, points or other using the disector tool by first selecting it in the

   "Z space" tab. Be sure to use the PEN tool
   a. Marks in the previous layer appear in red, and marks in the next layer appear in blue and marks in the current layer appear yellow.
   b. Useful keyboard shortcuts in the TrakEM2 environment
      i. <, > used to switch between layers
      ii. -, + to zoom in and zoom out
      iii. With the left mouse button:
         1. Click outside any existing marks to add a new mark, starting a new item to count.
         2. Click on the red or blue ghost of a mark to add a new mark for that item.
         3. Drag to move a mark. iv. With the click-wheel you can click and drag to navigate the current image layer or use the "Navigator Panel"

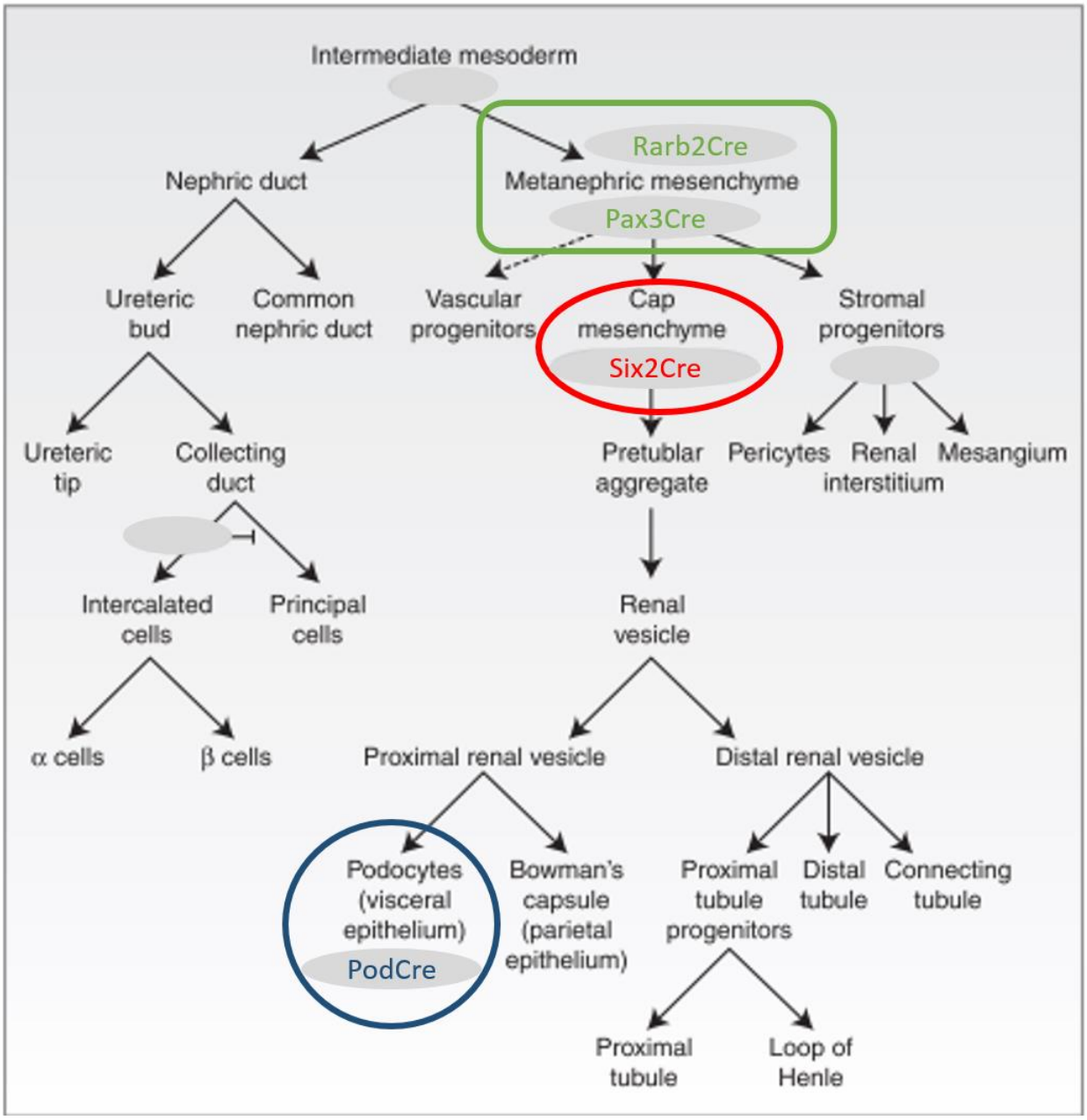


7. Generate reports



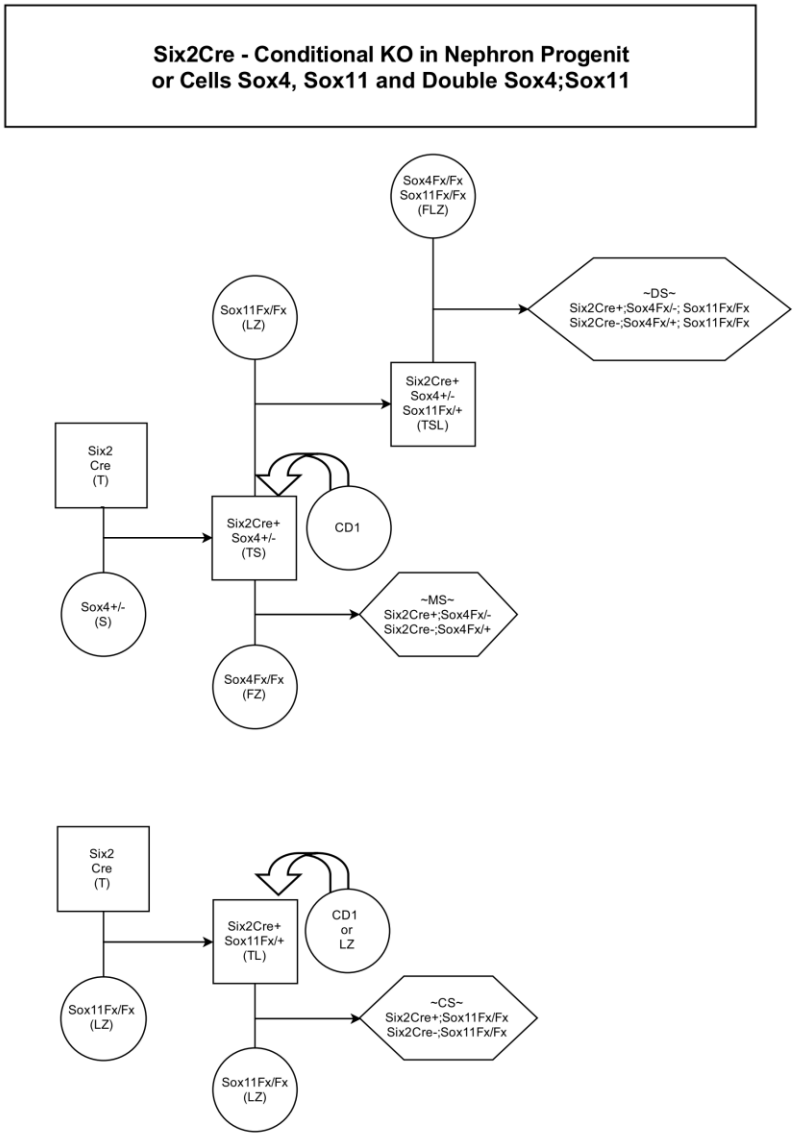
For more details and tutorials see the users manual of TrakEM2
http://www.ini.uzh.ch/~acardona/trakem2_manual.html

APPENDIX E – Target compartments of conditional knockout approaches. These conditional knockouts were attempted and generated, and indicated along with their derivatives. Adapted from Little and McMahon 2012.

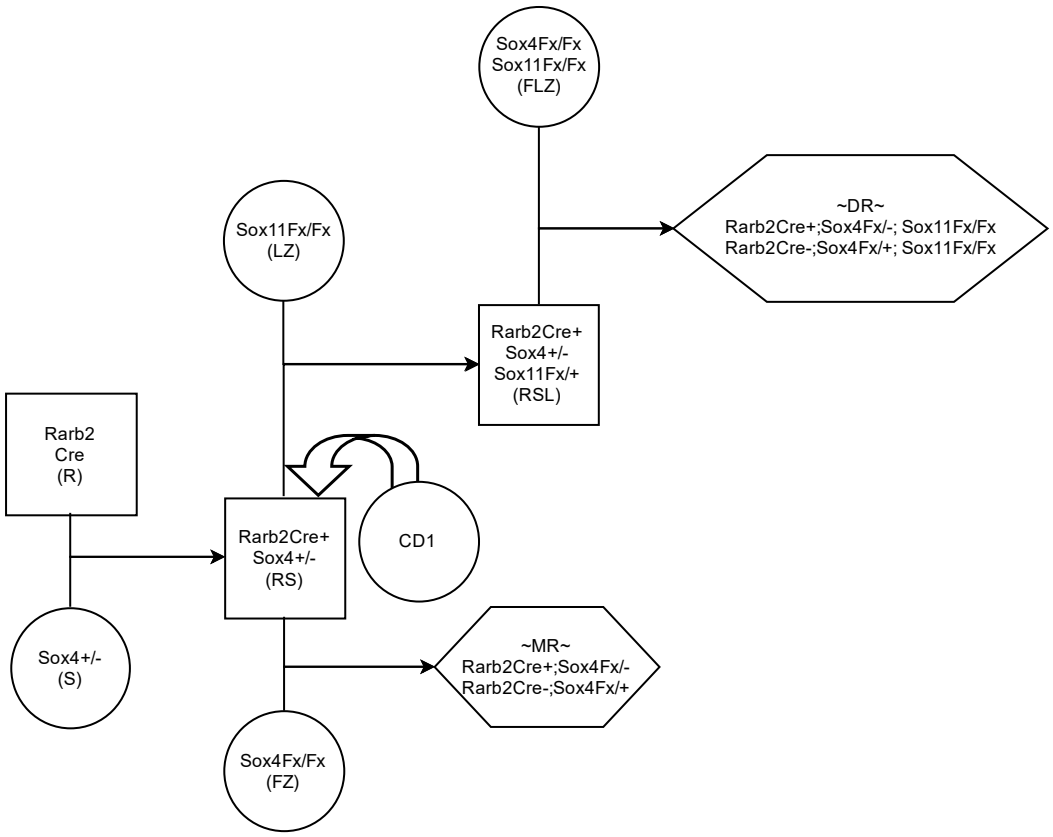


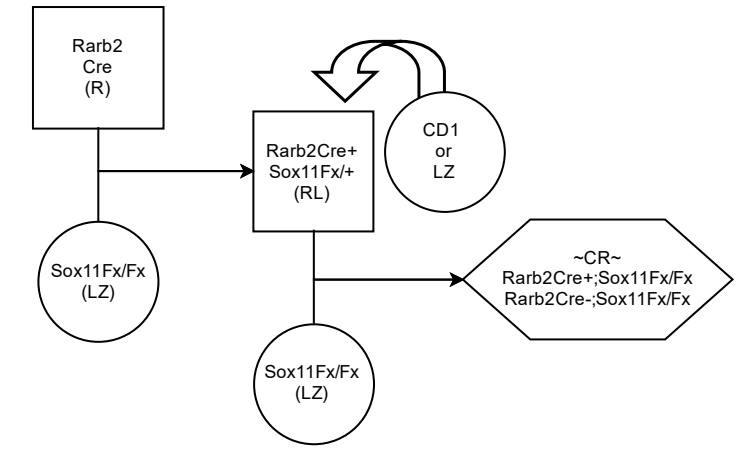
APPENDIX F - Mating schemes used to generate mouse models.

Circles represent females, squares represent males, elongated hexagons are desired experimental units. Curved arrows indicate maintenance matings.

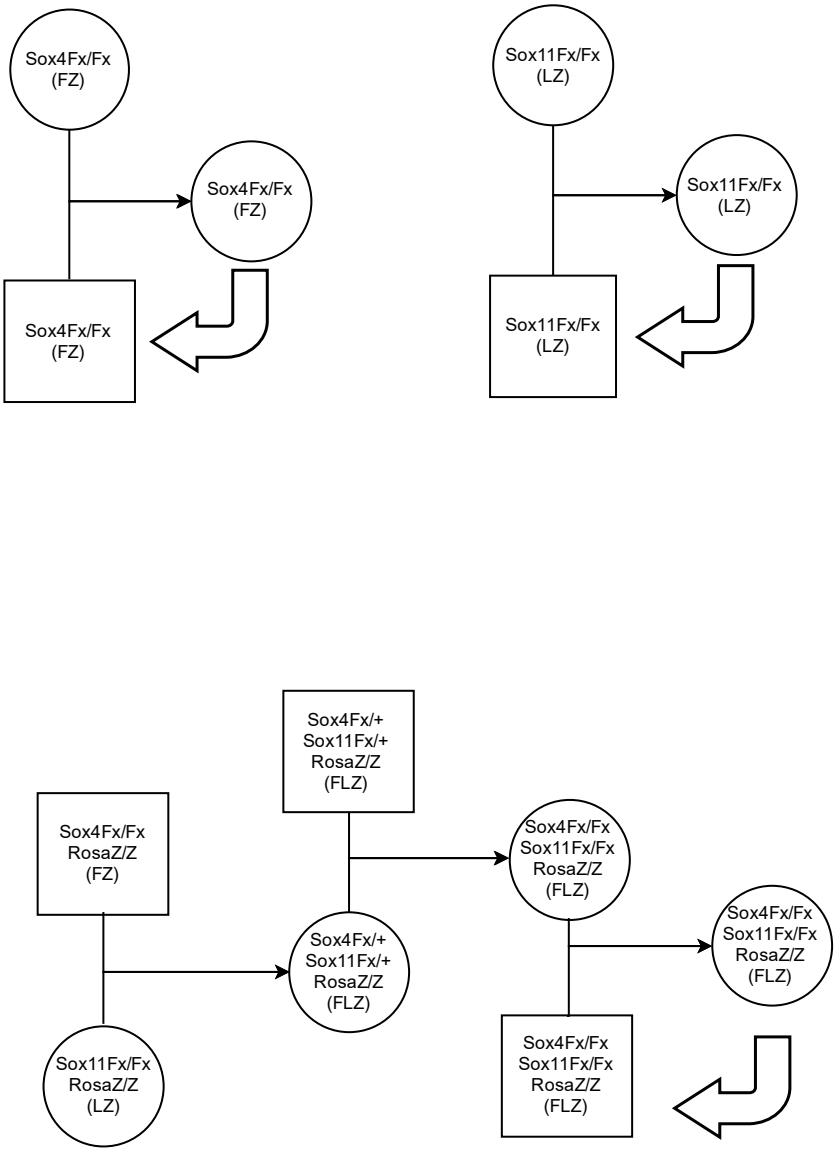


Rarb2Cre - Conditional KO in Nephrogenic Progenitors of the Metanephric Mesenchyme Sox4, Sox11 and Double Sox4;Sox11

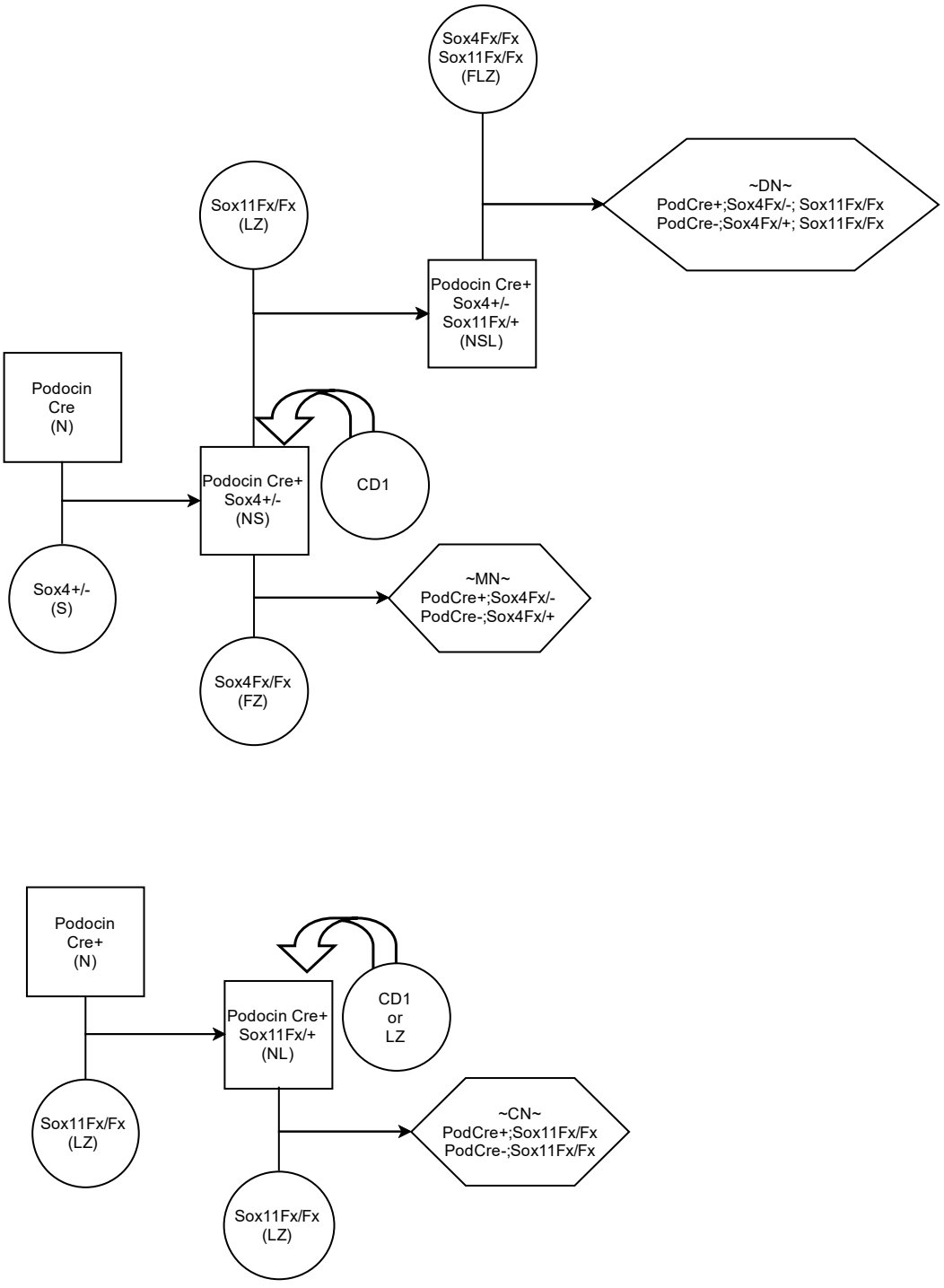




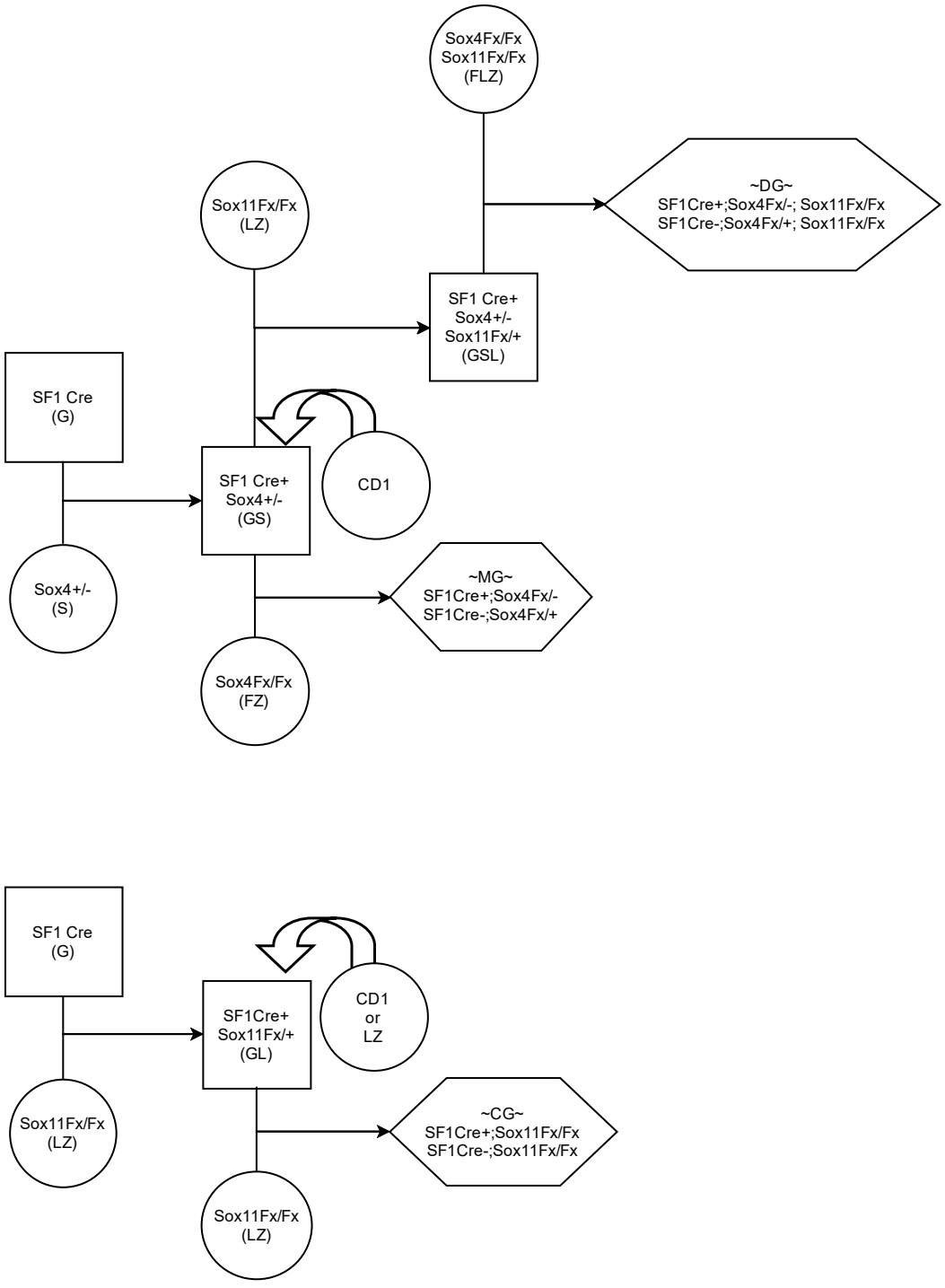
Generating Females for Crosses and Experimental Units



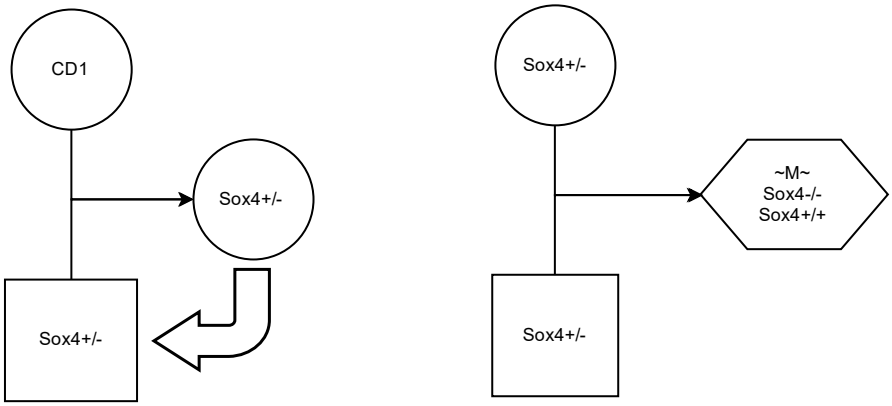
Podocin Cre - Conditional KO in Podocytes
Sox4, Sox11 and Double Sox4;Sox11

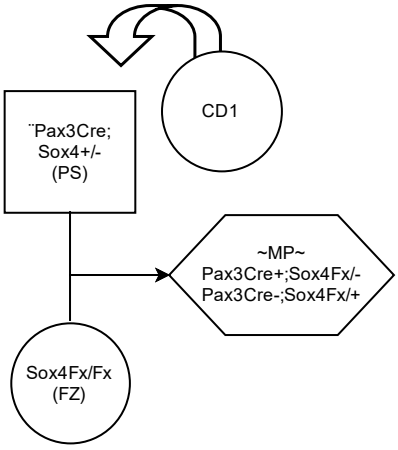


SF1Cre - Conditional KO in Gonads
Sox4, Sox11 and Double Sox4;Sox11



Sox4 Testes Phenotype





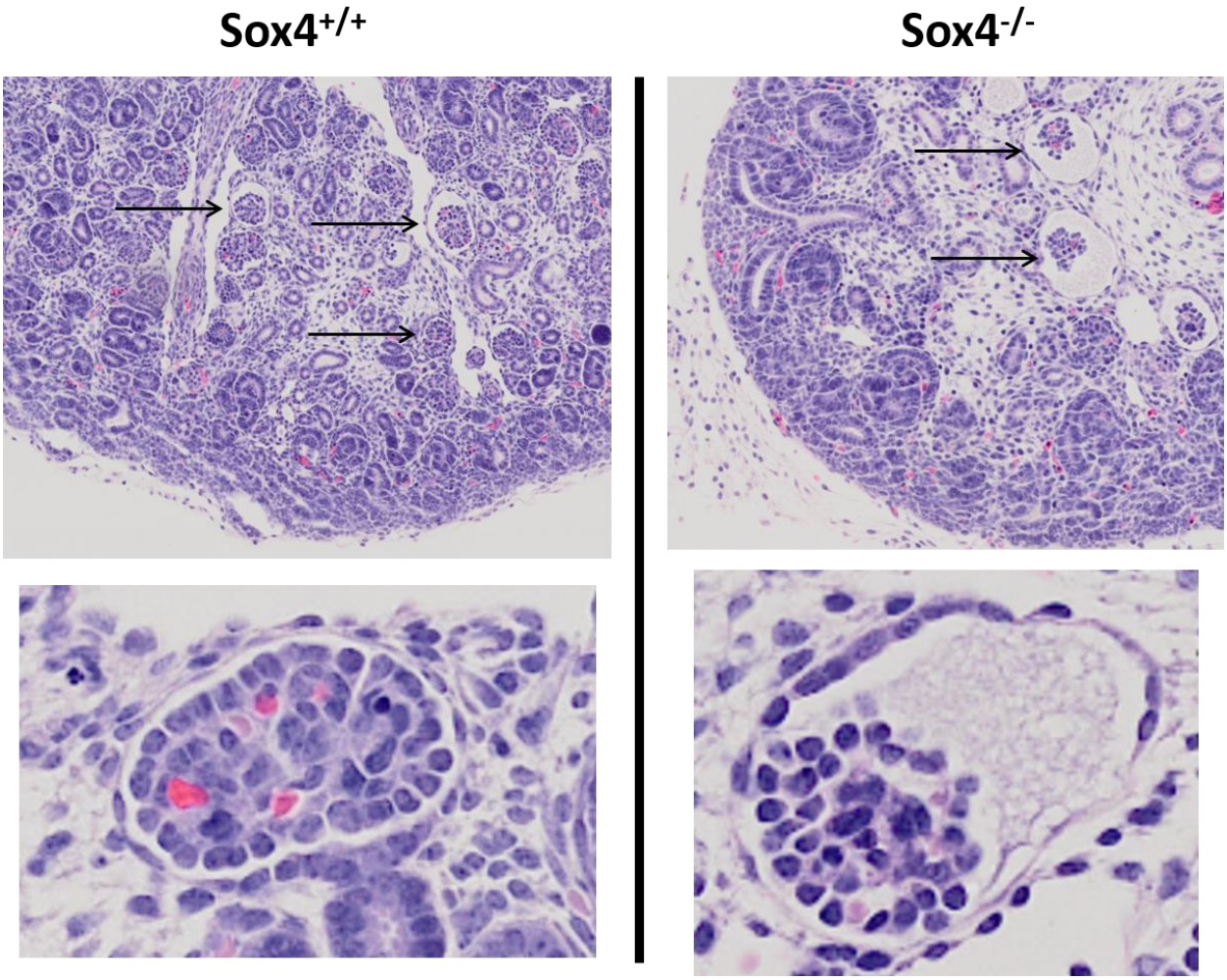
APPENDIX G – Summary table of mouse models evaluated over the course of the presented thesis

Mouse line and description	Target compartment	Objective	Brief summary of results & future work
Sox4+/-	N/A – Total KO	Evaluate the role of *Sox4* in the kidney prior to embryonic lethality.	In our hands the *Sox4-/-* embryos start resorbing at E14.5, by E15.5 no structures or cell types can be distinguished. Limited exploration of kidney development was carried out by examining embryonic kidneys stained for light microscopy. Some glomerular dilatation was observed in the KO compared to WT (See Appendix H). It is also in these animals that the elongated gonad phenotype was observed (see Appendix I) and a collaboration with Dr. Peter Koopman's group was established. Other simple experiments could be performed and comparison to Six2Cre conditional KO could further our understanding of mechanism such as e12.5 kidney explants for PTA counts and assessment of cell cycling.
Six2Cre	Nephron progenitor cells (NPC)	Evaluate the role of *Sox4* and *Sox11* in NPC	*Sox4* is essential for proper nephron endowment, and kidney development. In the absence of Sox4 in NPC, mice die of kidney failure. See Chapters 2, 3, and 4 for details.

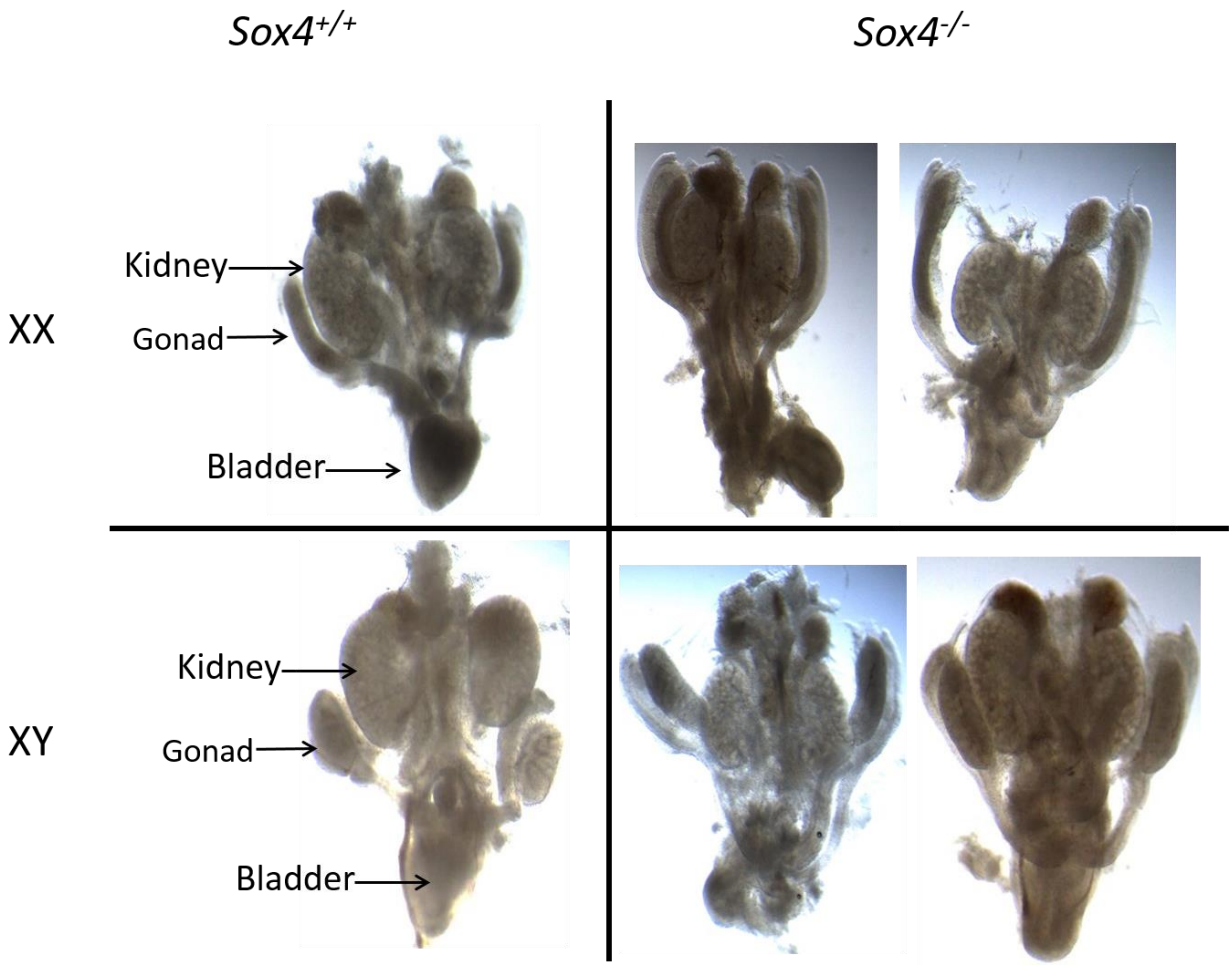
Pax3Cre	Metanephric mesenchyme	Evaluate the role of *Sox4* and *Sox11* in the metanephric mesenchyme (from E11.5) and its derivatives (NPC and stromal progenitors).	Crosses were started in conjunction with the Six2Cre crosses. Inconsistencies in phenotype penetrance and association with genotype was observed. Obtained 3 animals in long term study with unilateral hydronephrosis. Variations in phenotype possibly due to mosaicism of Cre expression. Once clear phenotype of SixCre model emerged, Pax3Cre was put to the side. Pax3 is also expressed at high levels in the genital ridges at E10.5. The elongated gonad phenotype observed in *Sox4-/-* embryos was recapitulated in the Pax3Cre model.
Rarb2Cre	Metanephric mesenchyme	Evaluate the role of *Sox4* and Sox11 in the metanephric mesenchyme (from E10.5) and its derivatives. Compare phenotype to that of Six2Cre.	Attempted for multiple generations to generate conditional KO of Sox4 using this Cre. Issues with litter size, consistently small consisting of only 2-4 very large pups, usually born after 20 days rather than 19. Outcrossed to CD1 for 3 generations to see if we could overcome the issue without any success. Personal communication with several individuals who have used the line confirmed that this is a known issue with the line, and it takes a long time to obtain sufficient animals. Following a cost benefit analysis decided to no longer maintain the line in-house.
PodCre	Podocytes	Evaluate the role of *Sox4* and Sox11 in podocytes.	The absence of *Sox4* in podocytes does not recapitulate the Six2 phenotype. See Chapter 4 for details.

SF1Cre	Somatic cells of gonads of both sexes	Attempt to recapitulate and further evaluate the role of *Sox4* in gonad development	At 6 weeks of age gonads appeared normal. As it is not the primary focus of our group, crosses were discontinued.

APPENDIX H – Glomerular dilatation is observed more frequently in Sox4-/- kidneys compared to wild-type control littermates at embryonic day (E)14.5 by H&E staining. Arrows indicate glomeruli (top), with higher magnification of a single glomeruli also shown (bottom).



APPENDIX I – Initial gonad elongation phenotype observed in Sox4-/- embryos at embryonic day (E)14.5. Phenotype was observed in both female (XX) and male (XY) embryos. The male phenotype was more actively pursued as it was more striking and resembled a possible ovotesti phenotype. The phenotype was also recapitulated with the Pax3Cre conditional KO of *Sox4*



APPENDIX J – In the absence of Sox4 there is an increase in gonad length while kidney size is maintained. A) representative images of male (XY) and female (XX) gonads and kidneys. B) Length measurements of fetal gonads indicate a significant increase in length for both male and female gonads. C) No significant difference is observed in length for male or female embryonic kidneys. Bar = 0.5mm. **P<0.01 (Liang Zhao)